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**10/591428****Plants with increased activity of a starch phosphorylating enzyme****5 Description**

The present invention relates to plant cells and plants that are genetically modified, whereby the genetic modification leads to an increase in the activity of a starch phosphorylating OK1 protein in comparison to corresponding wild type plant cells or wild type plants that have not been genetically modified. The present invention also relates to means and methods for the manufacture of such plant cells and plants. These types of plant cells and plants synthesise a modified starch. Therefore, the present invention also concerns the starch synthesised from the plant cells and plants according to the invention, methods for the manufacture of this starch, and the manufacture of starch derivatives of this modified starch, as well as flours containing starches according to the invention.

In addition, the present invention relates to nucleic acids, coding starch phosphorylating OK1 proteins, vectors, host cells, plant cells, and plants containing such nucleic acid molecules. The present addition also involves OK1 proteins, which exhibit starch-phosphorylating activity.

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With regard to the increasing importance currently attributed to plant constituents as renewable raw material sources, one of the tasks of biotechnological research is to endeavour to adapt these plant raw materials to suit the requirements of the processing industry. Furthermore, in order to enable regenerating raw materials to be used in as many areas of application as possible, it is necessary to achieve a large variety of materials.

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Polysaccharide starch is made up of chemically uniform base components, the glucose molecules, but constitutes a complex mixture of different molecule forms, which exhibit differences with regard to the degree of polymerisation and branching, and therefore differ strongly from one another in their physical-chemical characteristics. Discrimination is made between amylose starch, an essentially

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unbranched polymer made from alpha-1,4-glycosidically linked glucose units, and the amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional alpha-1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of  $5 \times 10^5$  -  $10^6$  Da, that of the amylopectin lies between  $10^7$  and  $10^8$ . The two macromolecules can be differentiated by their molecular weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

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Amylose has long been looked upon as a linear polymer, consisting of alpha-1,4-glycosidically linked alpha-D-glucose monomers. In more recent studies, however, the presence of alpha-1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

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The functional characteristics of starches, such as for example the solubility, the retrogradation behaviour, the water binding capacity, the film-forming characteristics, the viscosity, the gelatinisation characteristics, the freezing-thawing stability, the acid stability, the gel strength and the size of the starch grain, are affected amongst other things by the amylose/amylopectin ratio, the molecular weight, the pattern of the side chain distribution, the ion concentration, the lipid and protein content, the average grain size of the starch, the grain morphology of the starch etc. The functional characteristics of starch are also affected by the phosphate content, a non-carbon component of starch. Here, differentiation is made between phosphate, which is bonded covalently in the form of monoesters to the glucose molecules of the starch (described in the following as starch phosphate), and phosphate in the form of phospholipids associated with the starch.

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The starch phosphate content varies depending on the type of plant. Therefore, certain maize mutants, for example, synthesise a starch with increased starch phosphate content (waxy maize 0.002% and high-amylose maize 0.013%), while conventional types of maize only have traces of starch phosphate. Similarly small

amounts of starch phosphate are found in wheat (0.001%), while no evidence of starch phosphate has been found in oats and sorghum. Small amounts of starch phosphate have also been found in rice mutants (waxy rice 0.003%), and in conventional types of rice (0.013%). Significant amounts of starch phosphate have been shown in plants, which synthesise tuber or root storage starch, such as tapioca (0.008%), sweet potato (0.011%), arrowroot (0.021%) or potato (0.089%) for example. The percentage values for the starch phosphate content quoted above refer to the dry weight of starch in each case, and have been determined by Jane et al. (1996, Cereal Foods World 41 (11), 827-832).

Starch phosphate can be present in the form of monoesters at the C-2, C-3 or C-6 position of polymerised glucose monomers (Takeda and Hizukuri, 1971, Starch/Stärke 23, 267-272). The phosphate distribution of phosphate in starch synthesised by plants is generally characterised in that approximately 30% to 40% of residual phosphate at the C-3 position, and approximately 60% to 70% of the residual phosphate at the C-6 position, of the glucose molecule are covalently bonded (Blennow et al., 2000, Int. J. of Biological Macromolecules 27, 211-218). Blennow et al. (2000, Carbohydrate Polymers 41, 163-174) have determined a starch phosphate content, which is bonded in the C-6 position of the glucose molecules, for different starches such as, for example, potato starch (between 7.8 and 33.5 nMol per mg of starch, depending on the variety), starch from different *Curcuma* species (between 1.8 and 63 nMol per mg), tapioca starch (2.5 nMol per mg of starch), rice starch (1.0 nMol per mg of starch), mung bean starch (3.5 nMol per mg of starch) and sorghum starch (0.9 nMol per mg of starch). These authors have been unable to show any starch phosphate bonded at the C-6 position in barley starch and starches from different waxy mutants of maize. Up to now, it has not been possible to establish a connection between the genotype of a plant and the starch phosphate content (Jane et al., 1996, Cereal Foods World 41 (11), 827-832). It is therefore currently not possible to affect the starch phosphate content in plants by means of breeding measures.

Previously, only one protein has been described, which facilitates the introduction of covalent bonds of phosphate residues to the glucose molecules of starch. This

protein has the enzymatic activity of an alpha-glucan-water dikinase (GWD, E.C.: 2.7.9.4) (Ritte et al., 2002, PNAS 99, 7166-7171), is frequently described in the literature as R1, and is bonded to the starch grains of the storage starch in potato tubers (Lorberth et al., 1998, Nature Biotechnology 16, 473-477). In the reaction  
5 catalysed by R1, the educts alpha-1,4-glucan (starch), adenosintriphosphate (ATP) and water are converted to the products glucan-phosphate (starch phosphate), monophosphate and adenosine monophosphate. In doing so, the residual gamma phosphate of the ATP is transferred to water, and the residual beta phosphate of the ATP is transferred to the glucan (starch). R1 transfers the residual beta phosphate of  
10 ATP to the C-6 and the C-3 position of the glucose molecules of alpha-1,4-glucans *in vitro*. The ratio of C-6 phosphate to C-3 phosphate, which is obtained in the *in vitro* reaction, is the same as the ratio, which is present in starch isolated from plants (Ritte et al., 2002, PNAS 99, 7166-7171). As about 70% of the starch phosphate present in potato starch is bonded to the glucose monomers of the starch in the C-6  
15 position and about 30% in the C-3 position, this means that R1 preferably phosphorylates the C-6 position of the glucose molecules. Furthermore, it has been shown that by the use of amylopectin from maize, amongst other things, R1 can phosphorylate alpha-1,4-glucans, which do not yet contain covalently bonded phosphate (Ritte et al., 2002, PNAS 99, 7166-7171), i.e. R1 is able to introduce  
20 phosphate *de novo* into alpha-1,4-glucans.

Nucleic acid sequences, and the amino acid sequences corresponding to them, coding an R1 protein, are described from different species, such as, for example, potato (WO 97 11188, GenBank Acc.: AY027522, Y09533), wheat (WO 00 77229,  
25 US 6,462,256, GenBank Acc.: AAN93923, GenBank Acc.: AR236165), rice (GenBank Acc.: AAR61445, GenBank Acc.: AR400814), maize (GenBank Acc.: AAR61444, GenBank Acc.: AR400813), soya bean (GenBank Acc.: AAR61446, GenBank Acc.: AR400815), citrus (GenBank Acc.: AY094062) and *Arabidopsis* (GenBank Acc.: AF312027).

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Wheat plants, which exhibit increased activity of an R1 protein due to overexpression of an R1 potato gene, are described in WO 02 34923. These plants synthesise a starch with significant quantities of starch phosphate at the C-6 position of the

glucose molecules in comparison to corresponding wild type plants, in which no starch phosphate could be detected.

Further proteins, which catalyse a reaction, which introduce covalently bonded phosphate groups into the starch, have not previously been described. Enzymes, which preferably introduce phosphate groups in the C-3 position and/or the C-2 position of the glucose molecules of starch, are also unknown. Apart from the increase of the starch phosphate content in plants, there are therefore also no available ways of specifically influencing the phosphorylation of starch in plants, of modifying the phosphate distribution within the starch synthesised by plants and/or of further increasing the starch phosphate content.

The object of the present invention is therefore based on providing modified starches with increased phosphate content and/or modified phosphate distribution, as well as plant cells and/or plants, which synthesise such a modified starch, as well as means and methods for producing said plants and/or plant cells.

This problem is solved by the embodiments described in the claims.

The present invention therefore relates to genetically modified plant cells and genetically modified plants, characterised in that the plant cells or plants have an increased activity of at least one OK1 protein in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

A first aspect of the present invention relates to a plant cell or plant, which is genetically modified, wherein the genetic modification leads to an increase in the activity of at least one OK1 protein in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

At the same time, the genetic modification can be any genetic modification, which leads to an increase in the activity of at least one OK1 protein in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

In conjunction with the present invention, the term "wild type plant" means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

The term „increased activity of at least one OK1 protein" within the framework of the present invention means an increase in the expression of endogenous genes, which code the OK1 proteins, and/or an increase in the quantity of OK1 proteins in the cells, and/or an increase in the enzymatic activity of OK1 proteins in the cells.

The increase in the expression can be determined by measuring the quantity of OK1 proteins coding transcripts, for example; e.g. by way of Northern Blot analysis or RT-PCR. An increase preferably means an increase in the quantity of transcripts of at least 50%, preferably at least 70%, more preferably at least 85%, and most preferably at least 100%, in comparison to corresponding cells that have not been genetically modified. An increase in the quantity of transcripts coding an OK1 protein also means that plants or plant cells, which do not exhibit any detectable quantities of

transcripts coding an OK1 protein, show detectable quantities of transcripts coding an OK1 protein following genetic modification according to the invention.

5 The increase in the amount of protein of an OK1 protein, which results in increased activity of this protein in the plant cells concerned, can, for example, be determined by immunological methods such as Western blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio Immune Assay). Here, an increase preferably means an increase in the amount of OK1 protein in comparison with corresponding plant cells that have not been genetically modified by at least 50%, in particular by at  
10 least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the amount of OK1 protein also means that plants or plant cells that do not have any detectable OK1 protein activity exhibit a detectable quantity of OK1 protein following genetic modification according to the invention.

15 Methods for manufacturing antibodies, which react specifically with a certain protein, i.e. which bond specifically to said protein, are known to the person skilled in the art (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered by some companies (e.g. Eurogentec, Belgium) as a contract  
20 service. A possible way of manufacturing antibodies, which specifically react with an OK1 protein, is described below (see Example 10).

Within the framework of the present invention, the term "OK1 protein" is to be understood to mean a protein, which transfers a phosphate residue of ATP onto  
25 already phosphorylated starch (P-starch). Starches isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant have no detectable amounts of covalently bonded phosphate residues and are not phosphorylated by an OK1 protein, i.e. an OK1 protein according to the invention requires already phosphorylated starch as a substrate for transferring further phosphate residues.

30 Preferably, the residual beta phosphate of the ATP is transferred from an OK1 protein to the starch, and the residual gamma phosphate of the ATP is transferred to water. A further reaction product produced by a phosphorylating reaction of P-starch carried out using an OK1 protein is AMP (adenosine monophosphate). An OK1

protein is therefore described as [phosphorylated-alpha-glucan]-water-dikinase ([P-glucan]-water-dikinase) or as [phosphorylated-starch]-water-dikinase.

Preferably, an additional phosphate monoester bond is produced in the C-6 position and/or in the C-3 position of a glucose molecule of the P-starch, which is phosphorylated by an OK1 protein. In the phosphorylation of P-starch catalysed by an OK1 protein, it is particularly preferred if more additional phosphate monoester bonds are produced in the C-3 position in comparison with phosphate monoester bonds in the C-6 position of the glucose molecules of the P-starch concerned.

Amino acid sequences, which code OK1 proteins, contain a phosphohistidine domain. Phosphohistidine domains are described, for example, by Tien-Shin Yu et al. (2001, Plant Cell 13, 1907-1918). Phosphohistidine domains of OK1 proteins coding amino acids preferably contain two histidines.

In the catalysis of a phosphorylating reaction of a P-starch by means of an OK1 protein, a phosphorylated OK1 protein is produced as an intermediate product, in which a phosphate residue of ATP is covalently bonded to an amino acid of the OK1 protein. The intermediate product is produced by autophosphorylation of the OK1 protein, i.e. the OK1 protein itself catalyses the reaction, which leads to the intermediate product. Preferably, a histidine residue of the amino acid sequence coding an OK1 protein is phosphorylated as a result of the autophosphorylation process, particularly preferably a histidine residue, which is part of a phosphohistidine domain.

Furthermore, OK1 proteins according to the invention have an increased bonding activity to P-starch in comparison with non-phosphorylated starches.

As no enzymes have previously been described, which require P-starch as a substrate in order to phosphorylate them further, it has also previously not been possible to increase the starch phosphate content of already phosphorylated starch in plants above a certain quantity. This is now possible with the utilisation of a protein according to the invention or a nucleic acid molecule according to the invention for the genetic modification of plants. The clarification of the function of an OK1 protein, and thus the provision of an OK1 protein, leads to the fact that plants can now be genetically modified in such a way that they synthesise a starch with modified characteristics. The modification of the phosphate distribution in starch synthesised



by plants was previously not possible due to the lack of available means. Due to the provision by the present invention of proteins and nucleic acids according to the invention, it is now also possible to modify the phosphate ratio in native starches.

- 5 In conjunction with the present invention, the term "increased bonding activity" is to be understood to mean an increased affinity of a protein to a first substrate in comparison with a second substrate. That is to say, the amount of protein, which, under the same incubation conditions, bonds to a first substrate to a greater extent in comparison with a second substrate, exhibits increased bonding activity to the first  
10 substrate.

In conjunction with the present invention, the term "starch phosphate" is to be understood to mean phosphate groups covalently bonded to the glucose molecules of starch.

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- In conjunction with the present invention, the term "non-phosphorylated starch" is to be understood to mean a starch, which does not contain any detectable amounts of starch phosphate. Different methods of determining the amount of starch phosphate are described. Preferably, the method of determining the amount of starch phosphate  
20 described by Ritte et al. (2000, Starch/Stärke 52, 179-185) can be used. Particularly preferably, the determination of the amount of starch phosphate by means of <sup>31</sup>P-NMR is carried out according to the method described by Kasemusuwan and Jane (1996, Cereal Chemistry 73, 702-707).

- 25 In conjunction with the present invention, the term "phosphorylated starch" or "P-starch" is to be understood to mean a starch, which contains starch phosphate.

- The activity of an OK1 protein can be demonstrated, for example, by *in vitro* incubation of an OK1 protein using ATP, which contains a phosphate residue labeled  
30 in the beta position (labeled ATP). Preferably ATP is used, in which the phosphate residue is specifically labeled in the beta position, i.e. in which only the phosphate residue in the beta position has a marking. Preferably radioactively labeled ATP, particularly preferably ATP, in which the phosphate residue is specifically

radioactively labeled in the beta position, and especially preferably ATP, in which the phosphate residue in the beta position is specifically labeled with  $^{33}\text{P}$ , is used. If an OK1 protein with labeled ATP and starches, which are not phosphorylated, are incubated, no phosphate is transferred to the starch due to OK1. Preferably, leaf starch of *Arabidopsis thaliana* mutant *sex1-3* (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918) is used.

If, on the other hand, an OK1 protein with P-starch is incubated in the presence of labeled ATP, then labeled phosphate covalently bonded to the P-starch can subsequently be shown. Preferably, starch from leaves of *Arabidopsis thaliana*, particularly preferably starch from *Arabidopsis thaliana* *sex1-3* mutants enzymatically phosphorylated by means of an R1 protein (Ritte et al., 2002, PNAS 99, 7166-7171) is used.

Labeled phosphate residues, which have been incorporated in P-starch due to an OK1 protein, e.g. by separating the labeled P-starch (e.g. by precipitation with ethanol, filtration, chromatographic methods etc.) from the rest of the reaction mixture and subsequently detecting the labeled phosphate residue in the P-starch fraction, can be shown. At the same time, the labeled phosphate residues bonded in the P-starch fraction can be demonstrated, for example, by determining the amount of radioactivity present in the P-starch fraction (e.g. by means of scintillation counters).

Possible methods for demonstrating a protein, which requires P-starch as a substrate for a phosphorylating reaction, are described below under General Methods, Item 11 and in Example 6.

Which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in P-starch are preferably phosphorylated by an OK1 protein can be determined, for example, by analysing the P-starches phosphorylated by a protein, as described by Ritte et al. (2002, PNAS 99, 7166-7171). For this purpose, a P-starch phosphorylated by a protein is hydrolysed using an acid, and subsequently analysed by means of anion exchange chromatography.

Preferably, the P-starch phosphorylated by an OK1 protein is analysed by means of NMR in order to establish which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in the P-starch are phosphorylated. A particularly preferred method for identifying the C-atom positions of a glucose molecule of a starch, which

are phosphorylated by a reaction catalysed by an OK1 protein, is described below under General Methods, Item 13.

5 A phosphorylated protein, which is produced as an intermediate product in the phosphorylation of P-starch facilitated by an OK1 protein, can be demonstrated as described, for example, by Ritte et al. (2002, PNAS 99, 7166-7171) for an R1 protein.

To demonstrate the presence of an autophosphorylated intermediate product, an OK1 protein is first incubated in the absence of starch with labeled ATP, preferably  
10 with ATP specifically labeled in the beta phosphate position, particularly preferably with ATP specifically labeled with <sup>33</sup>P in the beta phosphate position. In parallel with this, a reaction preparation 2, which instead of labeled ATP contains corresponding amounts of non-labeled ATP however, is incubated under otherwise identical conditions. Subsequently, an excess of unlabeled ATP is added to reaction mixture 1  
15 and a mixture of unlabeled ATP and labeled ATP (the same quantity of labeled ATP as was used previously in reaction mixture 1, and the same quantity of the excess of unlabeled ATP that was added to reaction mixture 1) is added to reaction mixture 2, and this is further incubated before adding P-starch to Part A of reaction mixture 1 (Part 1A) and to Part A of reaction mixture 2 (Part 2A). The reaction in the remaining  
20 Part 1B and Part 2B of the reaction mixture is stopped by denaturing the protein. Part B of the reaction mixture can be stopped by the methods known to the person skilled in the art, which lead to the denaturing of proteins, preferably by adding sodium lauryl sulphate (SDS). Part 1A and Part 2A of the reaction mixture are incubated for at least a further 10 minutes before these reactions are also stopped. The starch present in  
25 Part A and Part B of the respective reaction mixture is separated from the remainder of the reaction mixture. If the respective starch is separated by centrifugation, for example, then, on completion of centrifugation, the starch of the respective Part A or Part B of the reaction mixture is to be found in the sedimented pellet, and the proteins in the respective reaction mixture are to be found in the supernatant of the  
30 respective centrifugation. The supernatant of Part 1A or 2A respectively and Part 1B or 2B respectively of the reaction mixture can subsequently be analysed by denaturing acrylamide gel electrophoresis, for example, followed by autoradiography of the acrylamide gel obtained. To quantify the amount of radioactively labeled

proteins, which have been separated by means of acrylamide gel electrophoresis, the so-called "phospho-imaging" method, for example, known to the person skilled in the art, can be used. If the autoradiography or the analysis by means of the "phospho-imager" of proteins in the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal compared with the centrifugation excess of Part A of reaction mixture 1, then this shows that a protein facilitating a phosphorylation of starch occurs as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal in the centrifugation supernatant in the autoradiography or in the analysis by means of the "phospho-imager".

In addition, the starch of the respective Part A of reaction mixture 1 and 2 remaining in the respective sedimented pellet can be investigated, if necessary after subsequent washing of the respective starches, for the presence of starch phosphate, which has a mark corresponding to the labeled ATP used. If the starches of Part A of reaction mixture 1 contain labeled phosphate residues, and if the autoradiography of the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal in the autoradiography compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a phosphorylation of starch-facilitating protein is present as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal for alpha-1,4-glucans labeled with  $^{33}\text{P}$  in the sedimented pellet containing alpha-1,4-glucans. Possible methods for demonstrating a phosphorylated OK1 protein intermediate product are described below under General Methods, Item 12 and in Example 7.

That an OK1 protein has an increased bonding activity to a P-starch compared with non-phosphorylated starch can be demonstrated by incubating the OK1 protein with P-starch and non-phosphorylated starch in separate preparations.

All non-phosphorylated starches are basically suitable for incubating OK1 proteins with non-phosphorylated starch. Preferably, a non-phosphorylated plant starch, particularly preferably wheat starch, and especially preferably granular leaf starch of an *Arabidopsis thaliana* mutant *sex1-3* is used.

Methods for isolating starch from plants, for example, are known to the person skilled in the art. All methods known to the person skilled in the art are basically suitable for isolating non-phosphorylated starch from appropriate plant species. Preferably, the methods for isolating non-phosphorylated alpha-1,4-glucans described below are  
5 used (see General Methods Item 2).

All starches, which contain starch phosphate, are basically suitable for incubating OK1 proteins with P-starch. Chemically phosphorylated starches can also be used for this purpose. Preferably, P-starches are used for the incubation with OK1  
10 proteins, particularly preferably a retrospectively enzymatically phosphorylated plant starch, especially preferably a retrospectively enzymatically phosphorylated plant granular starch, which has been isolated from a *sex-1* mutant of *Arabidopsis thaliana*.

To demonstrate an increased bonding activity of OK1 proteins to P-starch compared  
15 with non-phosphorylated starch, OK1 proteins are incubated in separate preparations with P-starch (Preparation A) and with non-phosphorylated starch (Preparation B). After successful incubation, the proteins, which are not bonded to the relevant starches of preparations A and B, are separated from the starches and the proteins to which they are bonded. The bond between the proteins and the P-starch in  
20 Preparation A and the bond between the proteins and non-phosphorylated starch in Preparation B are subsequently removed, i.e. the respective proteins are dissolved. The dissolved proteins of Preparation A and Preparation B can then be separated from the starches concerned, which are present in the respective preparations. Following this, the isolated P-starch bonding proteins of Preparation A and the  
25 isolated non-phosphorylated starch bonding proteins of Preparation B can be separated with the help of methods known to the person skilled in the art such as, for example, gel filtration, chromatographic methods, electrophoresis, SDS acrylamide gel electrophoresis etc. By comparing the amounts of separated proteins of Preparation A with the amounts of corresponding separated proteins of Preparation  
30 B, it can be determined whether a protein has an increased bonding activity with respect to P-starch compared with non-phosphorylated starch. Methods, which can be used to demonstrate a preferred bonding of proteins to P-starch compared with

non-phosphorylated starch, are described below in (General Methods, Item 8 and Example 1).

The amino acid sequence shown in SEQ ID NO 2 codes an OK1 protein from  
5 *Arabidopsis thaliana* and the amino acid sequence shown under SEQ ID NO 4 codes an OK1 protein from *Oryza sativa*.

In a further embodiment of the present invention, amino acid sequences coding an OK1 protein have an identity of at least 60% with the sequence specified in SEQ ID  
10 NO 2 or SEQ ID NO 4, in particular of at least 70%, preferably of at least 80% and particularly preferably of at least 90% and especially preferably of at least 95%.

In a further embodiment of the present invention, the OK1 protein exhibits a phosphohistidine domain (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918).  
15 Amino acid sequences coding OK1 proteins contain a phosphohistidine domain, which exhibits an identity of at least 50%, in particular of at least 60%, preferably of at least 70%, particularly preferably of at least 80%, and more particularly preferably of at least 90% of the amino acid sequence of the phosphohistidine domain of the OK1 protein from *Arabidopsis thaliana* and *Oryza sativa*, specified under SEQ ID NO  
20 5. The phosphohistidine domain preferably contains two histidines residues.

A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one  
25 foreign nucleic acid molecule into the genome of the plant.

In this context, the term "genetic modification" means the introduction of homologous and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to an  
30 increase in the activity of an OK1 protein.

The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign nucleic acid molecule. The presence or the expression of the foreign nucleic acid

molecule leads to a phenotypic change. Here, "phenotypic" change means preferably a measurable change of one or more functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit an increase in the activity of an  
5 OK1 protein due to the presence of or in the expression of the introduced nucleic acid molecule.

In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the  
10 corresponding wild type plant cells, or that does not occur naturally in the concrete spatial arrangement in wild type plant cells, or that is localised at a place in the genome of the plant cell at which it does not occur naturally in wild type plant cells. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of  
15 which does not occur naturally in plant cells.

In principle, the foreign nucleic acid molecule can be any nucleic acid molecule, which causes an increase in the activity of an OK1 protein in the plant cell or plant.

In conjunction with the present invention, the term "genome" is to be understood to  
20 mean the totality of the genetic material present in a plant cell. It is known to the person skilled in the art that, in addition to the cell nucleus, other compartments (e.g. plastids, mitochondria) also contain genetic material.

In a further embodiment, the plant cells according to the invention and the plants  
25 according to the invention are characterised in that the foreign nucleic acid molecule codes an OK1 protein, preferably an OK1 protein from *Arabidopsis thaliana* or an OK1 protein from *Oryza sativa*.

In a further embodiment, the foreign nucleic acid molecule codes an OK1 protein with  
30 the amino acid sequence specified in SEQ ID NO 2 or SEQ ID NO 4.

A large number of techniques are available for the introduction of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA

using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities.

The use of agrobacteria-mediated transformation of plant cells has been intensively investigated and adequately described in EP 120516; Hoekema, IN: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblaserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the potato transformation, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

The transformation of monocotyledonous plants by means of vectors based on *Agrobacterium* transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described in the literature many times (cf. e.g. WO95/06128, EP0513849, EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other types of cereal has also already been described, for example for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297; Becker et al., 1994, Plant Journal 5, 299-307). All the above methods are suitable within the framework of the present invention.



Amongst other things, plant cells and plants, which have been genetically modified by the introduction of an OK1 protein, can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that  
5 such a molecule is present integrated at a place in the genome of the plant cell according to the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type  
10 plants respectively in that they contain at least one copy of the foreign nucleic acid molecule stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of  
15 molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur (or they do not occur) in  
20 wild type plant cells or wild type plants. This can be verified, for example, with the help of a Southern blot analysis.

Furthermore, the plant cells according to the invention and the plants according to the invention can preferably be differentiated from wild type plant cells or wild type plants respectively by at least one of the following characteristics: If the foreign  
25 nucleic acid molecule that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-PCR (Reverse Transcription Polymerase Chain Reaction). Plant cells according to the invention and  
30 plants according to the invention, which express an antisense and/or an RNAi transcript, can be verified, for example, with the help of specific nucleic acid probes, which are complimentary to the RNA (occurring naturally in the plant cell), which is coding for the protein. Preferably, the plant cells according to the invention and the

plants according to the invention contain a protein, which is coded by an introduced nucleic acid molecule. This can be demonstrated by immunological methods, for example, in particular by a Western blot analysis.

5 If the foreign nucleic acid molecule that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain transcripts of the foreign nucleic acid  
10 molecules. This can be demonstrated by Northern blot analysis, for example, or with the help of so-called quantitative PCR.

15 In a further embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively.

In a further embodiment, the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of:

- 20 a) Nucleic acid molecules, which code a protein with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;
- b) Nucleic acid molecules, which code a protein, which includes the amino acid sequence, which is coded by the insertion in plasmid A.t.-OK1-pGEM or the insertion in plasmid pMI50;
- 25 c) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence given under SEQ ID NO 2 or SEQ ID NO 4;
- d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence, which is coded by the coding region of the insertion in plasmid A.t.-OK1-pGEM or by the coding region  
30 of the insertion in plasmid pMI50;
- e) Nucleic acid molecules, which include the nucleotide sequence shown under SEQ ID NO 1 or SEQ ID NO 3 or a complimentary sequence;

- f) Nucleic acid molecules, which include the nucleotide sequence of the insertion contained in plasmid A.t.-OK1-pGEM or plasmid pMI50;
- g) Nucleic acid molecules, which have an identity of at least 60% with the nucleic acid sequences described under a), b), e) or f);
- 5 h) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), d), e) or f) under stringent conditions;
- i) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), e) or f) due to the degeneration of the genetic code; and
- 10 j) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e), f), g), h) or i).

The amino acid sequence shown in SEQ ID NO 2 codes an OK1 protein from  
15 *Arabidopsis thaliana* and the amino acid sequence shown in SEQ ID NO 4 codes an OK1 protein from *Oryza sativa*.

The proteins coded from the different varieties of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example,  
20 biological activity, molecular weight, immunological reactivity, conformation etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc.

The molecular weight of the OK1 protein from *Arabidopsis thaliana* derived from the  
25 amino acid sequence shown under SEQ ID NO 2 is ca. 131 kDa and the molecular weight of the OK1 protein from *Oryza sativa* derived from the amino acid sequence shown under SEQ ID NO 4 is ca. 132 kDa. The derived molecular weight of a protein according to the invention therefore preferably lies in the range from 120 kDa to 145 kDa, preferably in the range from 120 kDa to 140 kDa, particularly preferably from  
30 125 kDa to 140 kDa and especially preferably from 130 kDa to 135 kDa.

The amino acid sequences shown in SEQ ID NO 2 and SEQ ID NO 4 coding OK1 proteins from *Arabidopsis thaliana* and *Oryza sativa* respectively each contain a phosphohistidine domain. Preferably, an OK1 protein according to the invention

therefore contains a phosphohistidine domain, which has an identity of at least 50%, preferably of at least 60%, particularly preferably of at least 80% and especially preferably of 90% with the phosphohistidine domain shown under SEQ ID NO 5.

- 5 The present invention relates to nucleic acid molecules, which code a protein with the enzymatic activity according to the invention of an OK1 protein, wherein the coded OK1 protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence specified under SEQ ID NO 2 or SEQ ID NO 4.

10

A plasmid (A.t.-OK1-pGEM) containing a cDNA which codes for a protein according to the invention (A.t.-OK1) from *Arabidopsis thaliana* was deposited on 08.03.2004 under the number DSM16264 and a plasmid (pMI50) containing a cDNA which codes for further protein according to the invention (O.s.-OK1) from *Oryza sativa* was  
15 deposited on 24.03.2004 under the number DSM16302 under the Budapest Treaty at the German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.

- The amino acid sequence shown in SEQ ID NO 2 can be derived from the coding region of the cDNA sequence integrated in plasmid A.t.-OK1-pGEM and codes for an  
20 OK1 protein from *Arabidopsis thaliana*. The amino acid sequence shown in SEQ ID NO 4 can be derived from the coding region of the cDNA sequence integrated in plasmid pMI50 and codes for an OK1 protein from *Oryza sativa*. The present invention therefore also relates to nucleic acid molecules, which code a protein with the enzymatic activity of an OK1 protein, which includes the amino acid sequence,  
25 which is coded by the insertion in plasmid A.t.-OK1-pGEM or by the insertion in plasmid pMI50, wherein the coded protein has an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of 95% with the amino acid sequence, which can be derived from the insertion in A.t.-OK1-pGEM or pMI50.

30

The nucleic acid sequence shown in SEQ ID NO 1 is a cDNA sequence, which includes the coding region for an OK1 protein from *Arabidopsis thaliana* and the

nucleic acid sequence shown in SEQ ID NO 3 is a cDNA sequence, which includes the coding region for an OK1 protein from *Oryza sativa*.

The present invention therefore also relates to nucleic acid molecules, which code an OK1 protein and the coding region of the nucleotide sequences shown under SEQ ID NO 1 or SEQ ID NO 3 or sequences, which are complimentary thereto, nucleic acid molecules, which include the coding region of the nucleotide sequence of the insertion contained in plasmid A.t.-OK1-pGEM or in plasmid pMI50 and nucleic acid molecules, which have an identity of at least 70%, preferably of at least 80%, particularly preferably of at least 90% and especially preferably of at least 95% with the said nucleic acid molecules.

With the help of the sequence information of nucleic acid molecules according to the invention or with the help of a nucleic acid molecule according to the invention, it is possible for the person skilled in the art to isolate homologous sequences from other plant species, preferably from starch-storing plants, preferably from plant species of the genus *Oryza*, in particular *Oryza sativa* or from *Arabidopsis thaliana*. This can be carried out, for example, with the help of conventional methods such as the examination of cDNA or genomic libraries with suitable hybridisation samples. The person skilled in the art knows that homologous sequences can also be isolated with the help of (degenerated) oligonucleotides and the use of PCR-based methods.

The examination of databases, such as are made available, for example, by EMBL (<http://www.ebi.ac.uk/Tools/index.htm>) or NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), can also be used for identifying homologous sequences, which code for OK1 protein. In this case, one or more sequences are specified as a so-called query. This query sequence is then compared by means of statistical computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers.

If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not

activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.

For nucleic acid sequence comparisons (blastn), the following parameters must be set: Limit entrez = not activated; Filter = low complexity activated; Expect value = 10;

5 word size = 11.

With such a database search, the sequences described in the present invention can be used as a query sequence in order to identify further nucleic acid molecules and/or proteins, which code an OK1 protein.

10 With the help of the described methods, it is also possible to identify and/or isolate nucleic acid molecules according to the invention, which hybridise with the sequence specified under SEQ ID NO 1 or under SEQ ID NO 3 and which code an OK1 protein.

Within the framework of the present invention, the term "hybridising" means hybridisation under conventional hybridisation conditions, preferably under stringent  
15 conditions such as, for example, are described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929). Particularly preferably, "hybridising" means hybridisation under the following conditions:

20 Hybridisation buffer:

2xSSC; 10xDenhardt solution (Ficoll 400+PEG+BSA; Ratio 1:1:1); 0.1% SDS; 5 mM EDTA; 50 mM Na<sub>2</sub>HPO<sub>4</sub>; 250 µg/ml herring sperm DNA; 50 µg/ml tRNA; or 25 M sodium-phosphate buffer pH 7.2; 1 mM EDTA; 7% SDS

Hybridisation temperature:

25 T=65 to 68°C

Wash buffer: 0.1xSSC; 0.1% SDS

Wash temperature: T=65 to 68°C.

30 In principle, nucleic acid molecules, which hybridise with the nucleic acid molecules according to the invention, can originate from any plant species, which codes an appropriate protein, preferably they originate from starch-storing plants, preferably from species of the (systematic) family *Poacea*, particularly preferably from *Oryza sativa*. Nucleic acid molecules, which hybridise with the molecules according to the

invention, can, for example, be isolated from genomic or from cDNA libraries. The identification and isolation of nucleic acid molecules of this type can be carried out using the nucleic acid molecules according to the invention or parts of these molecules or the reverse complements of these molecules, e.g. by means of

5 hybridisation according to standard methods (see, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929) or by amplification using PCR.

10 Nucleic acid molecules, which exactly or essentially have the nucleotide sequence specified under SEQ ID NO 1 or SEQ ID NO 3 or parts of these sequences, can be used as hybridisation samples. The fragments used as hybridisation samples can also be synthetic fragments or oligonucleotides, which have been manufactured using established synthesising techniques and the sequence of which corresponds

15 essentially with that of a nucleic acid molecule according to the invention. If genes have been identified and isolated, which hybridise with the nucleic acid sequences according to the invention, then a determination of this sequence and an analysis of the characteristics of the proteins coded by this sequence should be carried out in order to establish whether an OK1 protein is involved. Homology comparisons on the

20 level of the nucleic acid or amino acid sequence and a determination of the enzymatic activity are particularly suitable for this purpose. The activity of an OK1 protein can take place, for example, as described above under General Methods, Item 11. A preferred bonding affinity to P-starch in comparison with non-phosphorylated starch and autophosphorylation of an OK1 protein can be

25 demonstrated using the methods already described above and under General Methods, Items 8 and 12.

The molecules hybridising with the nucleic acid molecules according to the invention particularly include fragments, derivatives and allelic variants of the nucleic acid molecules according to the invention, which code an OK1 protein from plants,

30 preferably from starch-storing plants, preferably from plant species of the genus *Oryza*, particularly preferably from *Oryza sativa* or *Arabidopsis thaliana*. In conjunction with the present invention, the term "derivative" means that the sequences of these molecules differ at one or more positions from the sequences of

the nucleic acid molecules described above and have a high degree of identity with these sequences. Here, the deviation from the nucleic acid molecules described above can have come about, for example, due to deletion, addition, substitution, insertion or recombination.

5

In conjunction with the present invention, the term "identity" means a sequence identity over the whole length of the coding region of at least 60%, in particular an identity of at least 70%, preferably greater than 80%, particularly preferably greater than 90% and especially of at least 95%. In conjunction with the present invention,  
10 the term "identity" is to be understood to mean the number of amino acids/nucleotides (identity) corresponding with other proteins/nucleic acids, expressed as a percentage. Identity is preferably determined by comparing SEQ ID NO 2 or SEQ ID NO 4 for amino acids or SEQ. ID NO 1 or SEQ ID NO 3 for nucleic acids with other proteins/nucleic acids with the help of computer programs. If  
15 sequences that are compared with one another have different lengths, the identity is to be determined in such a way that the number of amino acids, which have the shorter sequence in common with the longer sequence, determines the percentage quotient of the identity. Preferably, identity is determined by means of the computer program ClustalW, which is well known and available to the public (Thompson et al.,  
20 Nucleic Acids Research 22 (1994), 4673-4680). ClustalW is made publicly available by Julie Thompson (Thompson@EMBL-Heidelberg.DE) and Toby Gibson (Gibson@EMBL-Heidelberg.DE), European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany. ClustalW can also be downloaded from different Internet sites, including the IGBMC (Institut de Génétique  
25 et de Biologie Moléculaire et Cellulaire, B.P.163, 67404 Illkirch Cedex, France; <ftp://ftp-igbmc.u-strasbg.fr/pub/>) and the EBI (<ftp://ftp.ebi.ac.uk/pub/software/>) as well as from all mirrored Internet sites of the EBI (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK).  
Preferably, Version 1.8 of the ClustalW computer program is used to determine the  
30 identity between proteins according to the invention and other proteins. In doing so, the following parameters must be set: KTUPLE=1, TOPDIAG=5, WINDOW=5, PAIRGAP=3, GAOPEN=10, GAPEXTEND=0.05, GAPDIST=8, MAXDIV=40, MATRIX=GONNET, ENDGAPS(OFF), NOPGAP, NOHGAP.



Preferably, Version 1.8 of the ClustalW computer program is used to determine the identity between the nucleotide sequence of the nucleic acid molecules according to the invention, for example, and the nucleotide sequence of other nucleic acid molecules. In doing so, the following parameters must be set:

5 KTUPLE=2, TOPDIAGS=4, PAIRGAP=5, DNAMATRIX:IUB, GAOPEN=10, GAPEXT=5, MAXDIV=40, TRANSITIONS: unweighted.

Furthermore, identity means that functional and/or structural equivalence exists between the nucleic acid molecules concerned or the proteins coded by them. The  
10 nucleic acid molecules, which are homologous to the molecules described above and constitute derivatives of these molecules, are generally variations of these molecules, which constitute modifications, which execute the same biological function. At the same time, the variations can occur naturally, for example they can be sequences from other plant species, or they can be mutants, wherein these mutants may have  
15 occurred in a natural manner or have been introduced by objective mutagenesis. The variations can also be synthetically manufactured sequences. The allelic variants can be both naturally occurring variants and also synthetically manufactured variants or variants produced by recombinant DNA techniques. Nucleic acid molecules, which deviate from nucleic acid molecules according to the invention due to degeneration of  
20 the genetic code, constitute a special form of derivatives.

The proteins coded from the different derivatives of nucleic acid molecules according to the invention have certain common characteristics. These can include, for example, biological activity, substrate specificity, molecular weight, immunological  
25 reactivity, conformation etc, as well as physical characteristics such as, for example, the running behaviour in gel electrophoresis, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic characteristics, stability; optimum pH, optimum temperature etc. Preferred characteristics of an OK1 protein have already been described in detail above and are to be applied here accordingly.

30

The nucleic acid molecules according to the invention can be any nucleic acid molecules, in particular DNA or RNA molecules, for example cDNA, genomic DNA, mRNA etc. They can be naturally occurring molecules or molecules manufactured by

genetic or chemical synthesis methods. They can be single-stranded molecules, which either contain the coding or the non-coding strand, or double-stranded molecules.

- 5 A further embodiment of the present invention relates to plant cells according to the invention and plants according to the invention wherein the foreign nucleic acid molecule is chosen from the group consisting of
- a) T-DNA molecules, which, due to integration into the plant genome, lead to an increase in the expression of at least one OK1 gene (T-DNA activation tagging);
  - 10 b) DNA molecules that contain transposons, which lead to an increase in the expression of an OK1 gene by way of integration into the plant genome. (transposon activation tagging);
  - c) DNA molecules that code an OK1 protein, and that are linked with regulatory sequences, which provide the transcriptions in plant cells, and which lead to an increase in the OK1 protein activity in the cell.
  - 15 d) Nucleic acid molecules introduced by means of *in vivo* mutagenesis, which lead to a mutation or an insertion of a heterologous sequence in at least one endogenous gene coding an OK1 protein, wherein the mutation or insertion causes an increase in the expression of a gene coding an OK1 protein.

20

In conjunction with the present invention, plant cells and plants according to the invention can also be manufactured by the use of so-called insertion mutagenesis (overview article: Thorneycroft et al., 2001, Journal of experimental Botany 52 (361), 1593-1601). Insertion mutagenesis is to be understood to mean particularly the

25 insertion of transposons or so-called transfer DNA (T-DNA) into a gene or near a gene coding for an OK1 protein, whereby, as a result of which, the activity of an OK1 protein in the cell concerned is increased.

The transposons can be both those that occur naturally in the cell (endogenous transposons) and also those that do not occur naturally in said cell but are introduced

30 into the cell (heterologous transposons) by means of genetic engineering methods, such as transformation of the cell, for example. Changing the expression of genes by means of transposons is known to the person skilled in the art. An overview of the use of endogenous and heterologous transposons as tools in plant biotechnology is

presented in Ramachandran and Sundaresan (2001, Plant Physiology and Biochemistry 39, 234-252).

T-DNA insertion mutagenesis is based on the fact that certain sections (T-DNA) of Ti plasmids from Agrobacterium can integrate into the genome of plant cells. The place  
5 of integration in the plant chromosome is not defined, but can take place at any point. If the T-DNA integrates into a part of the chromosome or near a part of the chromosome, which constitutes a gene function, then this can lead to an increase in the gene expression and thus also to a change in the activity of a protein coded by the gene concerned.

10 Here, the sequences inserted into the genome (in particular transposons or T-DNA) are distinguished by the fact that they contain sequences, which lead to an activation of regulatory sequences of an OK1 gene ("activation tagging").

Plant cells and plants according to the invention can be produced by means of the  
15 so-called "activation tagging" method (see, for example, Walden et al., Plant J. (1991), 281-288; Walden et al., Plant Mol. Biol. 26 (1994), 1521-1528). These methods are based on activating endogenous promoters by means of "enhancer" sequences, such as the enhancer of the 35S RNA promoter of the cauliflower mosaic virus, or the octopine synthase enhancer.

20

In conjunction with the present invention, the term "T-DNA activation tagging" is to be understood to mean a T-DNA fragment, which contains "enhancer" sequences and which leads to an increase in the activity of at least one OK1 protein by integration into the genome of a plant cell.

25

In conjunction with the present invention, the term "transposon activation tagging" is to be understood to mean a transposon, which contains "enhancer" sequences and which leads to an increase in the activity of at least one OK1 protein by integration into the genome of a plant cell.

30

In another embodiment, the DNA molecules according to the invention, which code an OK1 protein, are linked with regulatory sequences, which initiate transcription in plant cells (promoters) and lead to an increase in OK1 protein activity in the cell. In

this case, the nucleic acid molecules according to the invention are present in "sense" orientation to the regulatory sequences.

For expressing nucleic acid molecules according to the invention, which code an  
5 OK1 protein, these are preferably linked with regulatory DNA sequences, which guarantee transcription in plant cells. In particular, these include promoters. In general, any promoter that is active in plant cells is eligible for expression.

The promoter can be chosen so that expression takes place constitutively or only in a certain tissue, at a certain stage of the plant development or at a time determined by  
10 external influences. The promoter can be homologous or heterologous both with respect to the plant and with respect to the nucleic acid molecule.

Suitable promoters are, for example, the promoter of the 35S RNA of the cauliflower mosaic virus and the ubiquitin promoter from maize for constitutive expression, the patatin promoter B33 (Rocha-Sosa et al., EMBO J. 8 (1989), 23-29) for tuber-specific  
15 expression in potatoes or a promoter, which only ensures expression in photosynthetically active tissues, e.g. the ST-LS1 promoter (Stockhaus et al., Proc. Natl. Acad. Sci. USA 84 (1987), 7943-7947; Stockhaus et al., EMBO J. 8 (1989), 2445-2451) or, for endosperm-specific expression of the HMG promoter from wheat, the USP promoter, the phaseolin promoter, promoters of zein genes from maize  
20 (Pedersen et al., Cell 29 (1982), 1015-1026; Quatroccio et al., Plant Mol. Biol. 15 (1990), 81-93), glutelin promoter (Leisy et al., Plant Mol. Biol. 14 (1990), 41-50; Zheng et al., Plant J. 4 (1993), 357-366; Yoshihara et al., FEBS Lett. 383 (1996), 213-218) or shrunken-1 promoter (Werr et al., EMBO J. 4 (1985), 1373-1380). However, promoters can also be used, which are only activated at a time determined  
25 by external influences (see for example WO 9307279). Promoters of heat-shock proteins, which allow simple induction, can be of particular interest here. Furthermore, seed-specific promoters can be used, such as the USP promoter from Vicia faba, which guarantees seed-specific expression in Vicia faba and other plants (Fiedler et al., Plant Mol. Biol. 22 (1993), 669-679; Bäumlein et al., Mol. Gen. Genet.  
30 225 (1991), 459-467).

Furthermore, a termination sequence (polyadenylation signal) can be present, which is used for adding a poly-A tail to the transcript. A function in the stabilisation of the

transcripts is ascribed to the poly-A tail. Elements of this type are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged at will.

Intron sequences can also be present between the promoter and the coding region.

- 5 Such intron sequences can lead to stability of expression and to increased expression in plants (Callis et al., 1987, Genes Devel. 1, 1183-1200; Luehrsen, and Walbot, 1991, Mol. Gen. Genet. 225, 81-93; Rethmeier, et al., 1997; Plant Journal. 12(4):895-899; Rose and Beliakoff, 2000, Plant Physiol. 122 (2), 535-542; Vasil et al., 1989, Plant Physiol. 91, 1575-1579; XU et al., 2003, Science in China Series C
- 10 Vol.46 No.6, 561-569). Suitable intron sequences are, for example, the first intron of the sh1 gene from maize, the first intron of the polyubiquitin gene 1 from maize, the first intron of the EPSPS gene from rice or one of the two first introns of the PAT1 gene from Arabidopsis.
- 15 Furthermore, plant cells according to the invention and plants according to the invention can be manufactured by means of so-called "*in situ* activation". In this case, the introduced genetic modification effects a change in the regulatory sequences of endogenous OK1 genes, which leads to an increased expression of OK1 genes. Preferably, the activation of an OK1 gene takes place by "*in vivo*" mutagenesis of a
- 20 promoter or of "enhancer" sequences of an endogenous OK1 gene. In doing so, a promoter or an "enhancer" sequence, for example, can be changed in such a way that the mutation produced leads to an increased expression of an OK1 gene in plant cells according to the invention or plants according to the invention in comparison with the expression of an OK1 gene in wild type plant cells or wild type plants. The
- 25 mutation in a promoter or an "enhancer" sequence can also lead to OK1 genes in plant cells according to the invention or plants according to the invention being expressed at a time at which they would not be expressed in wild type plant cells or wild type plants.
- 30 In conjunction with the present invention, the term „OK1 gene“ is understood to mean a nucleic acid molecule (cDNA, DNA), which codes an OK1 protein, preferably an OK1 protein from starch-storing plants, more preferably from *Arabidopsis thaliana*, and most preferably from rice.

- During so-called "*in vivo*" mutagenesis, a hybrid RNA-DNA oligonucleotide („chimeroplast") is introduced into plant cells by way of transformation (Kipp, P.B. et al., Poster Session at the "5th International Congress of Plant Molecular Biology, September 21-27, 1997, Singapore; R. A. Dixon and C.J. Arntzen, Meeting report on "Metabolic Engineering in Transgenic Plants", Keystone Symposia, Copper Mountain, CO, USA, TIBTECH 15, (1997), 441-447; international patent WO 9515972; Kren et al., Hepatology 25, (1997), 1462-1468; Cole-Strauss et al., Science 273, (1996), 1386-1389; Beetham et al., 1999, PNAS 96, 8774-8778).
- 5 A part of the DNA components of the RNA-DNA oligonucleotide is homologous to a nucleic acid sequence of an endogenous OK1 gene, but, in comparison with the nucleic acid sequence of an endogenous OK1 gene, it has a mutation or contains a heterologous region, which is surrounded by the homologous regions.
- 10 By way of base pairing of the homologous regions of the RNA-DNA oligonucleotide and of the endogenous nucleic acid molecule, followed by homologous recombination, the mutation contained in the DNA component of the RNA-DNA oligonucleotide or heterologous region can be transferred into the genome of a plant cell. This leads to an increase in the activity of one or more OK1 proteins.
- 15
- 20 All these methods are based on the introduction of a foreign nucleic acid molecule into the genome of a plant cell or plant and are therefore basically suitable for the manufacture of plant cells according to the invention and plants according to the invention.
- 25 Surprisingly, it has been found that plant cells according to the invention and plants according to the invention synthesise a modified starch in comparison with starch of corresponding wild type plant cells or wild type plants that have not been genetically modified.
- 30 The plant cells according to the invention and plants according to the invention synthesise a modified starch, which in its physical-chemical characteristics, in particular the starch phosphate content or the phosphate distribution, is changed in

comparison with the synthesised starch in wild type plant cells or plants, so that this is better suited for special applications.

As no enzymes have previously been described, which exclusively phosphorylate P-starch, it has also previously not been possible to increase the starch phosphate content of already phosphorylated starch in plants over a certain level. This is now possible through the use of a protein according to the invention or a nucleic acid according to the invention for the genetic modification of plants.

It was not possible to distribute phosphates in starch synthesised from plants either, due to a lack of means available. Due to the provision of proteins and nucleic acids according to the present invention, it is now possible to alter the phosphate ratio in native starches as well.

Therefore, the present invention also includes plant cells and plants according to the invention, which synthesise a modified starch in comparison with corresponding wild type plant cells and wild type plants that have not been genetically modified.

In conjunction with the present invention, the term „modified starch“ should be understood to mean that the starch exhibits changed physical-chemical characteristics in comparison to unmodified starch, which is obtainable from corresponding wild type plant cells or wild type plants.

In an additional embodiment of the present invention, plant cells or plants according to the invention synthesise a starch, which contains a high content of starch phosphate and/or an altered phosphate distribution in comparison to starch that has been isolated from corresponding wild type plant cells and wild type plants.

In conjunction with the current invention, the term „phosphate distribution“ should be understood to mean the proportion of starch phosphate bonded to a glucose molecule in the C-2 position, C-3 position, or C-6 position, with respect to the total starch phosphate content in the starch.

In an additional embodiment of the present invention, plant cells or plants according to the invention synthesise a starch, which exhibits an altered ratio of C-3 phosphate to C-6 phosphate in comparison to starch from wild type plants that have not been genetically modified. Preferred here are starches, which have an increased  
5 proportion of starch phosphate bonded in the C-3 position compared with starch phosphate bonded in the C-6 position in comparison with starches from wild type plant cells and wild type plants that have not been genetically modified.

In conjunction with the present invention, the term "ratio of C-3 phosphate to C-6  
10 phosphate" should be understood to mean the amount of starch phosphate, of which starch phosphate bonded to an alpha-1,4-glucan in the C-3 position or C-6 position, respectively, contributes to the sum of the starch phosphate bonded to the alpha-1,4-glucan in the C-3 position and C-6 position (C-3 position + C-6 position).

15 Different methods of determining the amount of starch phosphate are described. Preferably, the method of determining the amount of starch phosphate described by Ritte et al. (2000, Starch/Stärke 52, 179-185) can be used. Particularly preferably, the determination of the amount of starch phosphate by means of  $^{31}\text{P}$ -NMR is carried out according to the method described by Kasemusuwan and Jane (1996,  
20 Cereal Chemistry 73, 702-707).

Furthermore, an object of the invention is genetically modified plants, which contain plant cells according to the invention. These types of plants can be produced from plant cells according to the invention by regeneration.

25

In principle, the plants according to the invention can be plants of any plant species, i.e. both monocotyledonous and dicotyledonous plants. Preferably they are useful plants, i.e. plants, which are cultivated by people for the purposes of food or for technical, in particular industrial purposes.

30

In a further embodiment, the plant according to the invention is a starch-storing plant.



In conjunction with the present invention, the term "starch-storing plants" means all plants with plant parts, which contain a storage starch, such as, for example, maize, rice, wheat, rye, oats, barley, cassava, potato, sago, mung bean, pea or sorghum.

- 5 In conjunction with the present invention, the term „potato plant“ or „potato“ means the plant species of the genus *Solanum*, particularly tuber-producing species of the genus *Solanum*, and in particular *Solanum tuberosum*.

- 10 In conjunction with the present invention, the term "wheat plant" means plant species of the genus *Triticum* or plants resulting from crosses with plants of the genus *Triticum*, particularly plant species of the genus *Triticum* or plants resulting from crosses with plants of the genus *Triticum*, which are used in agriculture for commercial purposes, and particularly preferably *Triticum aestivum*.

- 15 In conjunction with the present invention, the term "maize plant" means plant species of the genus *Zea*, particularly plant species of the genus *Zea*, which are used in agriculture for commercial purposes, particularly preferably *Zea mays*.

- 20 In an additional embodiment, the present invention relates to starch-storing plants according to the invention of the (systematic) family *Poaceae*. These are preferably maize or wheat plants.

The present invention also relates to propagation material of plants according to the invention containing a plant cell according to the invention.

25

- Here, the term "propagation material" includes those constituents of the plant that are suitable for producing offspring by vegetative or sexual means. Cuttings, callus cultures, rhizomes or tubers, for example, are suitable for vegetative propagation. Other propagation material includes, for example, fruits, seeds, seedlings, 30 protoplasts, cell cultures, etc. Preferably, the propagation material is tubers and particularly preferably grains, which contain endosperms.

In a further embodiment, the present invention relates to harvestable plant parts of plants according to the invention such as fruits, storage roots, roots, blooms, buds, shoots or stems, preferably seeds, grains or tubers, wherein these harvestable parts contain plant cells according to the invention.

5

Furthermore, the present invention also relates to a method for the manufacture of a genetically modified plant according to the invention, wherein

- a) a plant cell is genetically modified, whereby the genetic modification leads to an increase in the activity of an OK1 protein in comparison with corresponding wild  
10 type plant cells that have not been genetically modified;
- b) a plant is regenerated from plant cells from Step a); and
- c) if necessary, further plants are produced with the help of the plants according to Step b).

- 15 The genetic modification introduced into the plant cell according to Step a) can basically be any type of genetic modification, which leads to an increase in the activity of an OK1 protein.

The regeneration of the plants according to Step (b) can be carried out using methods known to the person skilled in the art (e.g. described in "Plant Cell Culture  
20 Protocols", 1999, ed. by R.D. Hall, Humana Press, ISBN 0-89603-549-2).

The production of further plants according to Step (c) of the method according to the invention can be carried out, for example, by vegetative propagation (for example using cuttings, tubers or by means of callus culture and regeneration of whole plants)  
25 or by sexual propagation. Here, sexual propagation preferably takes place under controlled conditions, i.e. selected plants with particular characteristics are crossed and propagated with one another. In this case, the selection is preferably carried out in such a way that further plants, which are obtained in accordance with Step c), exhibit the genetic modification, which was introduced in Step a).

30

In a further embodiment of the method according to the invention, the genetic modification consists in the introduction of a foreign nucleic acid molecule according to the invention into the genome of the plant cell, wherein the presence or the

expression of said foreign nucleic acid molecule leads to increased activity of an OK1 protein in the cell.

5 In a further embodiment of the method according to the invention, the genetic modification consists in the introduction of a foreign nucleic acid molecule into the genome of the plant cell, wherein the foreign nucleic acid molecule codes an OK1 protein.

10 In a further embodiment, the method according to the invention is used for manufacturing a genetically modified plant according to the invention for producing starch-storing plants.

In a further embodiment, the method according to the invention is used for producing maize or wheat plants according to the invention.

15

In a further embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

- a) Nucleic acid molecules, which code a protein with the amino acid sequence specified under SEQ ID NO 2 or SEQ ID NO 4;
- 20 b) Nucleic acid molecules, which code a protein that includes the amino acid sequence, which is coded by insertion into plasmid A.t.-OK1-pGE or insertion into plasmid pMI50;
- c) Nucleic acid molecules, which code a protein, the amino acid sequence of which has an identity of at least 60% with the amino acid sequence specified
- 25 under SEQ ID NO 2 or SEQ ID NO 4;
- d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% of the amino acid sequence that is coded by insertion into plasmid A.t.-OK1-pGEM or insertion into plasmid pMI50;
- e) Nucleic acid molecules, which include the nucleotide sequence shown under
- 30 SEQ ID NO 1 or SEQ ID NO 3 or a complimentary sequence;
- f) Nucleic acid molecules, which include the nucleotide sequence of insertion contained in the plasmid A.t.-OK1-pGEM or plasmid pMI50;

- g) Nucleic acid molecules, the nucleic acid sequence of which has an identity of at least 70% with the nucleic acid sequences described under a), b), e), or f);
- h) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), e), or f) under stringent conditions;
- 5 i) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules identified under a), b), e), or f) due to the degeneration of the genetic code, and
- j) Nucleic acid molecules, which represent fragments, allelic variants and/or derivatives of the nucleic acid molecules identified under a), b), c), d), e), f), g),  
10 h) or i).

In a further embodiment of the method according to the invention, the foreign nucleic acid molecule is chosen from the group consisting of

- 15 a) T-DNA molecules, which lead to an increase in the expression of an OK1 gene through integration into the plant genome (T-DNA activation tagging);
- b) DNA molecules, which contain transposons that lead to an increase in the expression of an OK1 gene through integration into the plant genome (transposon activation tagging);
- 20 c) DNA molecules, which code an OK1 protein and are linked to regulatory sequences that guarantee (initiate) the transcriptions in plant cells, and which lead to an increase in the activity of an OK1 protein in the cell;
- d) Nucleic acid molecules introduced by way of *in vivo* mutagenesis, which lead to a mutation or an insertion in a heterologous sequence in at least one endogenous OK1 gene, wherein the mutation or insertion causes an increase in  
25 the expression of an OK1 gene.

In a further embodiment, the present invention relates to a method according to the invention, wherein the genetically modified plant synthesises a modified starch in comparison with starch from wild type plants that have not been genetically modified.

30

In a further embodiment of the method according to the invention, the plants according to the invention synthesise a modified starch, which has a higher starch

phosphate content and/or a modified phosphate distribution in comparison with starch isolated from corresponding wild type plants.

5 In a further embodiment of the method according to the invention, the plants according to the invention synthesise a modified starch, which has a modified ratio of C-3 phosphate to C-6 phosphate in comparison with starch from wild type plants that have not been genetically modified. Particularly preferred here are starches, which have an increased proportion of starch phosphate bonded in the C-3 position compared with starch phosphate bonded in the C-6 position in comparison with  
10 starches from wild type plants that have not been genetically modified.

The present invention also relates to the plants obtainable by the method according to the invention.

15 Surprisingly, it has been found that starch isolated from plant cells according to the invention and plants according to the invention, which have an increased activity of an OK1 protein, synthesise a modified starch.

In particular, the increased quantities of starch phosphate in starches according to the invention provide the starches with surprising and advantageous properties.  
20 Starches according to the invention have an increased proportion of loaded groups due to the increased proportion of starch phosphate, which considerably affect the functional properties. Starch that contains loaded functional groups is particularly usable in the paper industry, where it is utilised for paper coating. Paper, which is coated with loaded molecules that also exhibit good adhesive properties, is  
25 particularly suitable for absorbing pigments, such as dye, printing inks, etc., for example.

The present invention also relates to modified starches obtainable from plant cells according to the invention or plants according to the invention, from propagation  
30 material according to the invention or from harvestable plant parts according to the invention.

In a further embodiment, the present invention relates to modified starch according to the invention from starch-storing plants, preferably from starch-storing plants of the (systematic) family Poaceae, particularly preferably from maize or wheat plants.

- 5 Furthermore the present invention relates to a method for the manufacture of a modified starch including the step of extracting the starch from a plant cell according to the invention or from a plant according to the invention, from propagation material according to the invention of such a plant and/or from harvestable plant parts according to the invention of such a plant, preferably from starch-storing parts
- 10 according to the invention of such a plant. Preferably, such a method also includes the step of harvesting the cultivated plants or plant parts and/or the propagation material of these plants before the extraction of the starch and, further, particularly preferably the step of cultivating plants according to the invention before harvesting.
- 15 Methods for extracting starches from plants or from starch-storing parts of plants are known to the person skilled in the art. Furthermore, methods for extracting starch from different starch-storing plants are described, e.g. in Starch: Chemistry and Technology (Publisher: Whistler, BeMiller and Paschall (1994), 2nd Edition, Academic Press Inc. London Ltd; ISBN 0-12-746270-8; see e.g. Chapter XII, Page
- 20 412-468: Maize and Sorghum Starches: Manufacture; by Watson; Chapter XIII, Page 469-479: Tapioca, Arrowroot and Sago Starches: Manufacture; by Corbishley and Miller; Chapter XIV, Page 479-490: Potato starch: Manufacture and Uses; by Mitch; Chapter XV, Page 491 to 506: Wheat starch: Manufacture, Modification and Uses; by Knight and Oson; and Chapter XVI, Page 507 to 528: Rice starch: Manufacture and
- 25 Uses; by Rohmer and Klem; Maize starch: Eckhoff et al., Cereal Chem. 73 (1996), 54-57, the extraction of maize starch on an industrial scale is generally achieved by so-called "wet milling".). Devices, which are in common use in methods for extracting starch from plant material are separators, decanters, hydrocyclones, spray dryers and fluid bed dryers.

30

In conjunction with the present invention, the term "starch-storing parts" is to be understood to mean such parts of a plant in which, in contrast to transitory leaf starch, starch is stored as a deposit for surviving for longer periods. Preferred starch-

storing plant parts are, for example, tubers, storage roots and grains, particularly preferred are grains containing an endosperm, especially particularly preferred are grains containing an endosperm of maize or wheat plants.

- 5 Modified starch obtainable by a method according to the invention for manufacturing modified starch is also the subject matter of the present invention.

In a further embodiment of the present invention, the modified starch according to the invention is native starch.

10

In conjunction with the present invention, the term "native starch" means that the starch is isolated from plants according to the invention, harvestable plant plants according to the invention, starch-storing parts according to the invention or propagation material of plants according to the invention by methods known to the person skilled in the art.

15

Furthermore, the use of plant cells according to the invention or plants according to the invention for manufacturing a modified starch are the subject matter of the present invention.

20

The person skilled in the art knows that the characteristics of starch can be changed by thermal, chemical, enzymatic or mechanical derivation, for example. Derived starches are particularly suitable for different applications in the foodstuffs and/or non-foodstuffs sector. The starches according to the invention are better suited to be an initial substance for the manufacture of derived starches than for conventional starches, since they exhibit a higher proportion of reactive functional groups due to the higher starch phosphate content.

25

The present invention therefore also relates to the manufacture of a derived starch, wherein modified starch according to the invention is derived retrospectively.

30

In conjunction with the present invention, the term "derived starch" is to be understood to mean a modified starch according to the invention, the characteristics

of which have been changed after isolation from plant cells with the help of chemical, enzymatic, thermal or mechanical methods.

5 In a further embodiment of the present invention, the derived starch according to the invention is starch that has been treated with heat and/or acid.

10 In a further embodiment, the derived starches are starch ethers, in particular starch alkyl ethers, O-allyl ethers, hydroxylalkyl ethers, O-carboxymethyl ethers, nitrogen-containing starch ethers, phosphate-containing starch ethers or sulphur-containing starch ethers.

In a further embodiment, the derived starches are cross-linked starches.

15 In a further embodiment, the derived starches are starch graft polymers.

In a further embodiment, the derived starches are oxidised starches.

20 In a further embodiment, the derived starches are starch esters, in particular starch esters, which have been introduced into the starch using organic acids. Particularly preferably these are phosphate, nitrate, sulphate, xanthate, acetate or citrate starches.

25 The derived starches according to the invention are suitable for different applications in the pharmaceutical industry and in the foodstuffs and/or non-foodstuffs sector. Methods for manufacturing derived starches according to the invention are known to the person skilled in the art and are adequately described in the general literature. An overview on the manufacture of derived starches can be found, for example, in Orthoefer (in Corn, Chemistry and Technology, 1987, eds. Watson und Ramstad, Chapter 16, 479-499).

30

Derived starch obtainable by the method according to the invention for manufacturing a derived starch is also the subject matter of the present invention.



Furthermore, the use of modified starches according to the invention for manufacturing derived starch is the subject matter of the present invention.

Starch-storing parts of plants are often processed into flours. Examples of parts of  
5 plants from which flours are produced, for example, are tubers of potato plants and  
grains of cereal plants. For the manufacture of flours from cereal plants, the  
endosperm-containing grains of these plants are ground and strained. Starch is a  
main constituent of the endosperm. In the case of other plants, which do not contain  
endosperm, and which contain other starch-storing parts instead such as tubers or  
10 roots, for example, flour is frequently produced by mincing, drying, and subsequently  
grinding the storing organs concerned. The starch of the endosperm or contained  
within starch-storing parts of plants is a fundamental part of the flour, which is  
produced from those plant parts, respectively. The characteristics of flours are  
therefore affected by the starch present in the respective flour. Plant cells according  
15 to the invention and plants according to the invention synthesise a modified starch in  
comparison with wild type plant cells and wild type plants that have not been  
genetically modified. Flours produced from plant cells according to the invention,  
plants according to the invention, propagation material according to the invention, or  
harvestable parts according to the invention, therefore exhibit modified properties.  
20 The properties of flours can also be affected by mixing starch with flours or by mixing  
flours with different properties.

Therefore, an additional subject of the invention relates to flours, which contain a  
starch according to the invention.

25

A further subject of the present invention relates to flours, which are produced from  
plant cells according to the invention, plants according to the invention, from starch-  
storing parts of plants according to the invention, from propagation material  
according to the invention, or from harvestable plant parts according to the invention.  
30 Preferred starch-storing parts of plants according to the invention are tubers, storage  
roots, and grains containing an endosperm. Tubers preferably come from potato  
plants, and grains preferably come from plants of the (systematic) family *Poaceae*,  
while grains particularly preferably come from maize or wheat plants.

In conjunction with the present invention, the term "flour" is to be understood to mean a powder obtained by grinding plant parts. Plant parts are possibly dried before grinding, and minced and/or strained after grinding.

5

Flours according to the invention are characterised in that they contain starch, which exhibits a modified phosphate content and/or a modified phosphate distribution particularly due to its increased water binding capacity. This is desirable in the processing of flours in the foodstuffs industry for many applications, and in particular in the manufacture of baked goods, for example.

10

A further subject of the present invention is a method for the manufacture of flours, including the step of grinding plant cells according to the invention, plants according to the invention, parts of plants according to the invention, starch-storing parts of plants according to the invention, propagation material according to the invention, or harvestable material according to the invention.

15

Flours can be produced by grinding starch-storing parts of plants according to the invention. Methods for the manufacture of flours are known to the person skilled in the art. A method for the manufacture of flours preferably includes the step of harvesting the cultivated plants or plant parts and/or the propagation material or the starch-storing parts of these plants before grinding, and particularly preferably includes the additional step of cultivating plants according to the invention before harvesting.

25

In conjunction with the present invention, the term „parts of plants“ should be understood to mean all parts of the plants that, as constituents, constitute a complete plant in their entirety. Parts of plants are scions, leaves, rhizomes, roots, knobs, tubers, pods, seeds, or grains.

30

In a further embodiment of the present invention, the method for the of flours includes processing plants according to the invention, starch-storing plants according to the

invention, propagation material according to the invention, or harvestable material according to the invention before grinding.

In this case, processing can be heat treatment and/or drying, for example. Heat treatment followed by a drying of the heat-treated material is used in the manufacture of flours from storage roots or tubers such as potato tubers, for example, before grinding. The mincing of plants according to the invention, starch-storing parts of plants according to the invention, propagation material according to the invention, or harvestable material according to the invention before grinding can also represent processing in the sense of the present invention. The removal of plant tissue before grinding, such as e.g. grain husks, also represents processing before grinding in the sense of the present invention.

In a further embodiment of the present invention, the method for the manufacture of flours includes processing the ground product after grinding.

In this case, the ground product can be strained after grinding, for example, in order to produce various types of flours, for example.

A further subject of the present invention is the use of genetically modified plant cells according to the invention or plants according to the invention for the manufacture of flours.

It is also an object of the present invention to provide means such as DNA molecules, for example, for the production of plant cells according to the invention and plants according to the invention, which synthesise a modified starch in comparison with modified wild type plant cells or wild type plants that have not been genetically modified.

The present invention therefore also relates to nucleic acid molecules, which code for a protein with the enzymatic activity of an OK1 protein, chosen from the group consisting of

- a) Nucleic acid molecules, which code a protein with the amino acid sequence specified under SEQ ID NO 2 or SEQ ID NO 4;

- b) Nucleic acid molecules, which code a protein that includes the amino acid sequence, which is coded by insertion into the plasmid A.t.-OK1-pGEM or insertion into the plasmid pMI50;
  - 5 c) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% with the amino acid sequence specified under SEQ ID NO 2 or SEQ ID NO 4;
  - d) Nucleic acid molecules, which code a protein, the sequence of which has an identity of at least 60% of the amino acid sequence, which is coded by insertion into the plasmid A.t.-OK1-pGEM or insertion into the plasmid DSM pMI50;
  - 10 e) Nucleic acid molecules, which include the nucleotide sequence specified under SEQ ID NO 1 or SEQ ID NO 3 or a complimentary sequence;
  - f) Nucleic acid molecules, which include the nucleotide sequence of the insertion contained in the plasmid A.t.-OK1-pGEM or the plasmid pMI50;
  - 15 g) Nucleic acid molecules, which have an identity of at least 70% with the nucleic acid sequences described under a), b), e), or f);
  - i) Nucleic acid molecules, which hybridise with at least one strand of the nucleic acid molecules described under a), b), e), or f) under stringent conditions;
  - h) Nucleic acid molecules, the nucleotide sequence of which deviates from the sequence of the nucleic acid molecules specified under a), b), e), or f) due to  
20 degeneration of the genetic code; and
  - j) Nucleic acid molecule, which represent fragments, allelic variants, and/or derivatives of the nucleic acid molecules specified under a), b), c), d), e), f), g), h), or i).
- 25 Basically, nucleic acid molecules according to the invention, can originate from any plant, preferably they originate from starch-storing plants, preferably from potato, barley, sorghum, barley, wheat, or rice plants, particularly preferably from *Arabidopsis* plants or rice plants, and more particularly preferably from *Oryza sativa*.
- 30 Furthermore, the present invention relates to nucleic acid molecules of at least 21, preferably more than 50 and particularly preferably more than 200 nucleotides length, which specifically hybridise with at least one nucleic acid molecule according to the invention. Here, specifically hybridise means that these molecules hybridise with

nucleic acid molecules, which code a protein according to the invention, but not with nucleic acid molecules, which code other proteins. In particular, the invention relates to such nucleic acid molecules, which hybridise with transcripts of nucleic acid molecules according to the invention and, as a result, can hinder their translation.

- 5 Such nucleic acid molecules, which specifically hybridise with the nucleic acid molecules according to the invention, can, for example, be constituents of antisense, RNAi or co-suppression constructs or ribozymes, or can be used as primers for PCR amplification.
- 10 Furthermore, the invention relates to recombinant nucleic acid molecules containing a nucleic acid molecule according to the invention.

In conjunction with the present invention, the term „recombinant nucleic acid molecule“ is to be understood to mean a nucleic acid molecule, which contains  
15 additional sequences in addition to nucleic acid molecules according to the invention, which do not naturally occur in the combination in which they occur in recombinant nucleic acids according to the invention. Here, the abovementioned additional sequences can be any sequences, preferably they are regulatory sequences (promoters, termination signals, enhancers), particularly preferably they are  
20 regulatory sequences that are active in plant tissue, and especially particularly preferably they are regulatory sequences that are active in plant tissue, in which storage starch is synthesised. Methods for the creation of recombinant nucleic acid molecules according to the invention are known to the person skilled in the art, and include genetic methods such as bonding nucleic acid molecules by way of ligation,  
25 genetic recombination, or new synthesis of nucleic acid molecules, for example (see e.g. Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929).

30

A further embodiment of recombinant nucleic acid molecules of the present invention are vectors, in particular plasmids, cosmids, viruses, bacteriophages, and other

customary vectors in gene technology, which contain the nucleic acid molecules according to the invention described above.

In a further embodiment, the nucleic acid molecules according to the invention  
5 contained in the vectors are linked with regulatory sequences, which initiate expression in prokaryotic or eukaryotic cells. Here, the term „expression“ can mean both transcription and translation. The nucleic acid molecules according to the invention can have an in “sense” orientation and/or an „antisense“ orientation with respect to the regulatory sequences.

10

Regulatory sequences for expression in prokaryotic organisms, e.g. *E. coli*, and in eukaryotic organisms are sufficiently described in literature, in particular such for expression in yeast are described, such as e.g. *Saccharomyces cerevisiae*. An overview of various systems for expression for proteins in various host organisms  
15 can be found, for example, in Methods in Enzymology 153 (1987), 383-516 and in Bitter et al. (Methods in Enzymology 153 (1987), 516-544).

A further subject of the present invention is a host cell, particularly a prokaryotic or eukaryotic cell, which is genetically modified with a nucleic acid molecule according  
20 to the invention and/or with a vector according to the invention, as well as cells that originate from these types of host cells, and which contain the genetic modification according to the invention.

In a further embodiment, the invention relates to host cells, particularly prokaryotic or  
25 eukaryotic cells, which were transformed with a nucleic acid molecule according to the invention or with a vector according to the invention, as well as host cells, which originate from these types of host cells, and which contain the described nucleic acid molecules according to the invention or vectors.

30 The host cells can be bacteria cells (e.g. *E. coli*, bacteria of the genus *Agrobacterium*, particularly *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*) or fungal cells (e.g. yeast, particularly *S. cerevisiae*, *Agaricus*, in particular *Agaricus bisporus*, *Aspergillus*, *Trichoderma*), as well as plant or animal cells. Here, the term

„transforms“ means that the cells according to the invention are genetically modified with a nucleic acid molecule according to the invention, inasmuch as they contain at least one nucleic acid molecule according to the invention in addition to their natural genome. This can occur in the cell freely, possibly as a self-replicating molecule, or it  
5 can be stably integrated into the genome of the host cell.

The host cells of microorganisms are preferable. Within the framework of the present patent application, this is understood to include all bacteria and all protists (e.g. fungi, particularly yeasts and algae), as they are defined in Schlegel "General Microbiology"  
10 (Georg Thieme Publishing House (1985), 1-2), for example.

Further host cells according to the invention are plant cells. In principle, these can be plant cells from any plant species, i.e. both monocotyledonous and dicotyledonous plants. These are preferably plant cells from agricultural useful plants, i.e. from plants, which are cultivated by humans for nutritional, technical, or particularly  
15 industrial purposes. The invention relates preferably to plant cells and plants from starch-storing plants (maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum); in particular, plant cells from plants of the (systematic) family *Poacea*, particularly preferably plant cells from maize or wheat plants.

20 Compositions containing a nucleic acid molecule according to the invention, recombinant nucleic acid molecule according to the invention or a vector according to the invention are also the subject matter of the present invention. Compositions containing a nucleic acid molecule according to the invention, a recombinant nucleic acid molecule according to the invention, or a vector according to the invention, and  
25 a host cell are preferred. Particularly preferably, the host cell is a plant cell, more particularly preferably a cell from maize or wheat plants.

A further aspect of Compositions according to the invention relates to compositions, which can be used for producing host cells according to the invention, preferably for  
30 producing plant cells according to the invention. Preferably, this is a composition containing a nucleic acid molecule according to the invention, a recombinant nucleic acid molecule according to the invention, or a vector according to the invention, and a biolistic carrier, which is suitable for the introduction of a nucleic acid molecule

according to the invention into a host cell. Preferred biolistic carriers are particles of tungsten, gold or synthetic materials.

A further embodiment of compositions according to the invention relates to compositions containing a nucleic acid molecule according to the invention, a recombinant nucleic acid molecule according to the invention, or a vector according to the invention, and a plant cell and a synthetic cultivation medium. Preferably, such compositions also contain polyethylene glycol (PEG) in addition to nucleic acid molecules according to the invention, plant cells, and a synthetic cultivation medium.

10 In the case of these compositions, the recombinant nucleic acid molecule according to the invention occurs outside of the plant cell, i.e. it is located outside of the cell interior of the plant cell, which is enclosed by a cytoplasmic membrane.

Synthetic culture media, which are suitable for the cultivation and/or transformation of plant cells, are known to the person skilled in the art, and are sufficiently described in literature, for example. Many different synthetic cultivation media are also available for purchase in the specialised trade (e.g. DUCHEFA Biochemie B.V., Belgium).

A further embodiment of compositions according to the invention relates to compositions, which are used for the identification of nucleic acids according to the invention. Preferably, such compositions contain additional nucleic acid molecules, in addition to a nucleic acid molecule according to the invention, a recombinant nucleic acid molecule according to the invention, or a vector according to the invention, particularly nucleic acid molecules of plant origination, which can occur in the form of genomic DNA, mRNA, or as clones in so-called DNA libraries. DNA libraries, which occur as cosmids, phagmids, plasmids, YACs or BACs are preferred. The DNA libraries can contain both genomic DNA and cDNA. The nucleic acid molecules according to the invention, recombinant nucleic acid molecules according to the invention, or a vector according to the invention are used in these compositions, preferably as a hybridisation sample.

30

A further embodiment of the present invention relates to a protein, which exhibits starch-phosphorylating activity, and which requires phosphorylated starch as a



substrate. Preferably, this is a protein, which exhibits phosphorylated starch phosphorylating activity, and which requires phosphorylated starch as a substrate.

5 A further embodiment of the present invention relates to a protein according to the invention, which requires phosphorylated starch as a substrate, and transfers a residual phosphate of ATP to phosphorylated starch. Preferably, a protein according to the invention transfers the residual beta-phosphate of ATP to phosphorylated starch. Particularly preferably, a protein according to the invention transfers the residual beta-phosphate of the ATP to phosphorylated starch and the residual  
10 gamma-phosphate of ATP to water, and therefore possesses the activity of a [phosphorylated-alpha-1,4-glucan]-water-dikinase or a [phosphorylated-starch]-water-dikinase.

15 A further embodiment of the present invention relates to a protein according to the invention, which accumulates as a phosphorylated intermediate product when transferring residual phosphate to phosphorylated starch.

A further embodiment of the present invention relates to a protein according to the invention, which exhibits increased bonding activity to phosphorylated starch in  
20 comparison to non-phosphorylated starch.

A further embodiment of the present invention relates to a protein according to the invention, which introduces more additional phosphate monoester bonds in the C-3 position in comparison to phosphate monoester bonds in the C-6 position of the  
25 glucose molecules of a phosphorylated starch.

Preferably, at least 30%, more preferably at least 60%, particularly preferably at least 90%, and most preferably at least 120% more phosphate monoester bonds in the C-3 position of the glucose molecules of a phosphorylated starch are introduced in comparison with the phosphate monoester bonds in the C-6 position of the glucose  
30 molecules of a phosphorylated starch.

A further subject of the present invention relates to a protein according to the invention, which exhibits a molecular weight derived from the amino acid sequence of

120 kDa to 145 kDa, preferably from 120 kDa to 140 kDa, particularly preferably from 125 kDa to 140 kDa, and most particularly preferably from 130 kDa to 135 kDa.

5 A further embodiment of the present invention relates to a protein according to the invention, which exhibits a phosphohistidine domain. The phosphohistidine domain preferably contains two residual histidines.

A further subject of the present invention is proteins according to the invention chosen from the group consisting of

- 10 a) Proteins, which include the amino sequence specified under SEQ ID NO 2 or SEQ ID NO 4;
- b) Proteins, which are coded by the coding region of the DNA inserted into the plasmid A.t.-OK1-pGEM or pMI50; or
- 15 c) Proteins, which exhibit an identity of at least 60% with the amino acid sequence of the proteins specified under a) or b).

In a further embodiment, the present invention relates to proteins with phosphorylated starch phosphorylating activity, wherein the coded protein exhibits an identity of at least 70%, preferably at least 80%, particularly preferably at least 90%,  
20 and more particularly preferably at least 95% with the amino acid sequence specified under SEQ ID NO 2 or SEQ ID NO 4, or with the amino acid sequence of an OK1 protein coded by the insertion into plasmid A.t.-OK1-pGEM or plasmid pMI50.

A further embodiment of the present invention relates to a protein according to the  
25 invention, characterised in that the amino acid sequence coding the protein exhibits a phosphohistidine domain. Preferably, the protein according to the invention exhibits a phosphohistidine domain, which has an identity of at least 50%, particularly at least 60%, preferably at least 70%, particularly preferably at least 80%, and more particularly preferably at least 90% with the amino acid sequence specified in SEQ ID  
30 NO 5.

In a further embodiment, the present invention relates to a protein according to the invention, wherein the protein originates from an *Arabidopsis* or a rice plant.

A further embodiment of the present invention relates to a protein, which exhibits increased bonding activity to phosphorylated starch in comparison with non-phosphorylated starch, wherein the bonding activity to phosphorylated starch is increased by at least three times, preferably at least four times, particularly preferably at least five times, and more particularly preferably at least six times, in comparison to the bonding activity of a non-phosphorylated starch.

In a further embodiment, the invention also relates to proteins, which are coded by nucleic acid molecules according to the invention.

### Description of Sequences

SEQ ID NO 1: Nucleic acid sequence comprising the coding region of the A.t.-OK1 proteins from *Arabidopsis thaliana*. This sequence is inserted in the A.t.-OK1-pGEM and OK1-pDEST17 vectors.

SEQ ID NO 2: Amino acid sequence coding the A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence can be derived from the nucleic acid sequence specified under SEQ ID NO 1.

SEQ ID NO 3: Nucleic acid sequence comprising the coding region of the O.s.-OK1 protein from *Oryza sativa*. This sequence is inserted in the pMI50 vector.

SEQ ID NO 4: Amino acid sequence coding the O.s.-OK1 protein from *Oryza sativa*. This sequence can be derived from the nucleic acid sequence specified under SEQ ID NO 3.

SEQ ID NO 5: Peptide sequence coding the phosphohistidine domain of the OK1 proteins from *Arabidopsis thaliana* and *Oryza sativa*.

### Description of Figures

Fig. 1: Denaturing acrylamide gel for identifying proteins from *Arabidopsis thaliana*, which preferably bond to non-phosphorylated starch in comparison with phosphorylated starch. A standard protein molecular weight marker is

shown in trace "M". Proteins obtained after incubating control preparation C from Example 1 d) are shown in trace "-". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with non-phosphorylated starch, isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant (Preparation B, example 1 d), are shown in trace "K". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with starch, isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation A, Example 1 d), are shown in trace "P". On completion of electrophoresis, the acrylamide gel was stained with Coomassie Blue.

Fig. 2: Demonstration of autophosphorylation of the OK1 protein. Fig. 2 A) shows a denaturing (SDS) acrylamide gel on completion of electrophoresis stained with Coomassie Blue. Fig. 2 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. M: Standard protein molecular weight marker; R1: Sample from reaction vessel 1 according to Example 7 (after incubating an OK1 protein with ATP); R2: Sample from reaction vessel 2 according to Example 7 (after incubating an OK1 protein with ATP the protein was heated to 95°C); R3: Sample from reaction vessel 3 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M HCl); R4: Sample from reaction vessel 4 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M NaOH).

Fig. 3: Demonstration of the starch-phosphorylating activity of an OK1 protein (see Example 6). OK1 protein was incubated with non-phosphorylated starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant (Preparation A) and starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation B). Preparation C is the same as Preparation B, except that this Preparation C was incubated without OK1 protein. Two independent tests were carried out for each preparation (A, B, C) (Test 1 and Test 2). The respective amounts are shown graphically, measured in cpm (counts per minute), on <sup>33</sup>P labeled phosphate,

which were introduced into non-phosphorylated starch (Preparation A) and phosphorylated starch (Preparation B) by the OK1 protein

Fig. 4: Comparison of the C-atom positions of glucose molecules of the starch, which was phosphorylated from an R1 protein and an OK1 protein respectively (see Example 9). OK1 protein (Preparation A) was incubated in the presence of ATP labeled with  $^{33}\text{P}$  with starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein. R1 protein (Preparation B) was incubated in the presence of ATP labeled with  $^{33}\text{P}$  with starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant. On completion of incubation, a total hydrolysis of the starch was carried out and the hydrolysis products were separated by means of HPAE chromatography. As standard, glucose-6-phosphate and glucose-3-phosphate were added to the hydrolysis products before separation. The hydrolysis products separated by means of HPAE chromatography were collected in individual fractions. The added glucose-6-phosphate eluted with fraction 15 and the added glucose-3-phosphate with fraction 17. The fractions obtained were subsequently investigated for the presence of radioactively labeled phosphate. The amount of  $^{33}\text{P}$  labeled phosphate measured in the individual fractions, measured in cpm (counts per minute), which was introduced into the hydrolysis products of the phosphorylated starch by the OK1 protein or the R1 protein, is shown graphically.

Fig. 5 Demonstration of the autophosphorylation of the OK1 protein. Fig. 5 A) shows a Western blot. Fig. 5 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. The OK1 protein was incubated either with randomised radioactively labeled ATP or with ATP specifically radioactively labeled in the gamma position. On completion of incubation, the proteins were either heated to 30°C or 95°C, or incubated in 0.5 M NaOH or 0.5 M HCl respectively.

Fig. 6 Demonstration of the transfer of the beta-phosphate residue of ATP to starch in a reaction catalysed by an OK1 protein. Either ATP specifically labeled

with  $^{33}\text{P}$  in the gamma position or randomised  $^{33}\text{P}$  ATP was used to phosphorylate starch, which had been phosphorylated *in vitro* by means of an R1 protein and isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, by means of an OK1 protein. No OK1 protein was added in any of the experiments designated as "control". Each preparation was tested twice, independently from one another. The results of both tests are shown.

Fig. 7 Western Blot analysis of protein extracts from plants using an antibody against the OK1 protein from *Arabidopsis thaliana*. Protein extracts from leaves of the following plants are shown: Ara *Arabidopsis thaliana*; 51, 54, 55, 67, 72, 73, 79, 62, 63, 64, 65, 69, 66, 68 are independent lines of the transformation 385JH; D wildtype *Solanum tuberosum* cv Désirée.

## General Methods

In the following, methods are described, which can be used for carrying out methods described in the invention. These methods constitute specific embodiments of the present invention but do not restrict the present invention to these methods. The person skilled in the art knows that he can implement the invention in the same way by modifying the methods described and/or by replacing individual parts of the methods by alternative parts of the methods.

### 1. Manufacture of Protein Extracts from Plant Tissue

#### a) Manufacture of protein extracts from plant tissue

Leaf material is frozen in liquid nitrogen immediately after harvesting, and subsequently homogenised in the mortar under liquid nitrogen. The reduced leaf material is mixed with ca. 3.5-times the volume (with respect to the weight of the leaf material used) of cold (4°C) binding buffer and broken down for 2 x 10 s with an Ultraturrax (maximum speed). After the first treatment with an Ultraturrax, the reduced leaf material is cooled on ice before the second treatment is carried out. The

treated leaf material is then passed through a 100- $\mu$ m nylon mesh and centrifuged for 20 min (50 ml centrifuge vessel, 20.000xg, 4°C).

b) Precipitation of the proteins contained in the protein extracts

- 5 The supernatant obtained following centrifugation according to Step a) is removed and its volume determined. To precipitate proteins, ammonium sulphate is added continuously to the supernatant over a period of 30 minutes while stirring on ice down to a final concentration of 75% (weight/volume). The supernatant is subsequently incubated for a further hour on ice while stirring. The proteins precipitated from the
- 10 supernatant are pelleted at 20.000xg and 4°C for 10 min and the pellets are subsequently absorbed in 5 ml of binding buffer, i.e. the proteins present in the pellet are dissolved.

c) Desalting of the precipitated proteins

- 15 The dissolved proteins are desalted by means of a PD10 column filled with Sephadex G25 (Amersham Bioscience, Freiburg, Prod. No. columns: 17-0851-01, Prod. No. Sephadex G25-M: 17-0033-01) at a temperature of 4°C, i.e. the ammonium sulphate used under Step b) for precipitation is also separated from the dissolved protein. The PD10 column is equilibrated with binding buffer before the
- 20 proteins dissolved in accordance with Step b) are applied. For this purpose, 5 ml of binding buffer are spread over the column in each case. Subsequently, 2.5 ml of the protein solution obtained in accordance with Step b) are added to each column before proteins are eluted from the column with 3.5 ml binding buffer.

25 d) Determination of the protein concentration

The protein concentration is determined with a Bradford assay (Biorad, Munich, Prod. No. 500-0006 (Bradford, 1976, Anal. Biochem. 72, 248-254)).

e) Composition of the binding buffer [

- 30 Binding buffer:      50 mM      HEPES/NaOH (or KOH), pH 7.2  
                             1 mM      EDTA  
                             2 mM      Dithioerythritol (DTE)  
                             2 mM      Benzamidine

2 mM	$\epsilon$ -Aminocaproic acid
0.5 mM	PMSF
0.02 %	Triton X-100

## 2. Isolation of Leaf Starch

- 5 a) Isolation of starch granules from plant tissues
- Leaf material is frozen immediately after harvesting in liquid nitrogen. The leaf material is homogenised in portions in the mortar under liquid nitrogen and absorbed into a total of ca. 2.5-times the volume (weight/volume) of starch buffer. In addition, this suspension is again homogenised in the Waring blender for 20 s at maximum
- 10 speed. The homogenate is passed through a nylon mesh (100  $\mu$ m mesh width) and centrifuged for 5 minutes at 1.000xg. The supernatant with the soluble proteins is discarded.
- b) Cleaning the starch isolated from the plant tissues
- 15 After removing the green material lying on top of the starch by rinsing off the green material with starch buffer, the pellet containing the starch obtained from Step a) is absorbed in starch buffer and successively passed through nylon meshes with different mesh widths (in the order of 60  $\mu$ m, 30  $\mu$ m, 20  $\mu$ m). The filtrate is centrifuged using a 10 ml Percoll cushion (95% (v/v) Percoll (Pharmacia, Uppsala,
- 20 Sweden), 5% (v/v) 0.5M HEPES-KOH pH7.2) (Correx tube, 15 min, 2.000xg). The sediment obtained after this centrifugation is re-suspended once in starch buffer and centrifuged again (5 min, 1.000xg).
- c) Removal of the proteins bonded to the starch
- 25 Following Step b), starch granules are obtained, which contain proteins bonded to the starch. The proteins bonded to the surface of the starch granules are removed by incubating four times with 0.5 % SDS (sodium lauryl sulphate) for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 5.000xg), in order to separate the starch granules from the
- 30 respective wash buffer.
- d) Purification of starch that has been freed of proteins



The starch obtained from Step c), which has been freed from the proteins bonded to its surface, is subsequently removed by incubating four times with wash buffer for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 5.000xg), in order to separate the starch granules from the respective wash buffer. These cleaning steps serve mainly to remove the SDS used in the incubations in Step c).

e) Determination of the concentration of isolated starch

The amount of starch isolated in Step d) is determined photometrically. After suitable dilution, the optical density of the starch suspension is measured against a calibration curve at a wavelength of 600 nm. The linear range of the calibration curve is located between 0 and 0.3 extinction units.

To produce the calibration curves, starch, for example isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, is dried under vacuum, weighed and absorbed in a defined volume of water. The suspension so obtained is diluted with water in several steps in a ratio of 1 to 1 in each case until a suspension of ca. 5 µg starch per ml of water is obtained. The suspensions obtained by the individual dilution steps are measured in the photometer at a wavelength of 600 nm. The absorption values obtained for each suspension are plotted against the concentration of starch in the respective suspension. The calibration curve obtained should follow a linear mathematical function in the range from 0 µg starch per ml of water to 0.3 µg starch per ml of water.

f) Storage of isolated starch

The starch can either be used directly without further storage for further tests, or stored in aliquots in 1.5 mL Eppendorf vessels at -20°C. Both the frozen starch and the non-stored, freshly isolated starch can be used, if required, for the methods described in the present invention relating to *in vitro* phosphorylation and/or binding test, for example.

g) Composition of buffers used

1x starch buffer: 20 mM HEPES-KOH, pH 8.0  
0.2 mM EDTA

0.5 % Triton X-100

Wash buffer: 50 mM HEPES/KOH, pH 7,2

5    **3. Recombinant Expression of an Identified Starch-phosphorylating Protein**

a)    Manufacture of a bacterial expression vector containing a cDNA, which codes a starch-phosphorylating protein

The cDNA coding a starch-phosphorylating protein can be amplified, for example, using mRNA or poly-A-plus-mRNA from plant tissues as a "template", by means of a  
10    polymerase chain reaction (PCR). For this purpose, a reverse transcriptase is first used for the manufacture of a cDNA strand, which is complementary to an mRNA, which codes a starch-phosphorylating protein, before the cDNA strand concerned is amplified by means of DNA polymerase. So-called "kits" containing substances, enzymes and instructions for carrying out PCR reactions are available for purchase  
15    (e.g. SuperScript™ One-Step RT-PCR System, Invitrogen, Prod. No.: 10928-034). The amplified cDNA coding a starch-phosphorylating protein can subsequently be cloned in a bacterial expression vector, e.g. pDEST™ (17 (Invitrogen). pDEST™17 contains the T7 promoter, which is used to initiate the transcription of the T7-RNA-polymerase. Furthermore, the expression vector pDEST™17 contains a Shine  
20    Dalgarno sequence in the 5'-direction of the T7 promoter followed by a start codon (ATG) and by a so-called His tag. This His tag consists of six codons directly following one another, which each code the amino acid histidine and are located in the reading frame of the said start codon. The cloning of a cDNA coding a starch-phosphorylating protein in pDEST™17 is carried out in such a way that a translational  
25    fusion occurs between the codons for the start codon, the His tag and the cDNA coding a starch-phosphorylating protein. As a result of this, following transcription initiated on the T7 promoter, and subsequent translation, a starch-phosphorylating protein is obtained, which contains additional amino acids containing the His tag on its N-terminus.  
30    However, other vectors, which are suitable for expression in microorganisms, can also be used for the expression of a starch-phosphorylating protein. Expression vectors and associated expression strains are known to the person skilled in the art

and are also available for purchase from the appropriate dealer in suitable combinations.

b) Manufacture of expression clones in *Escherichia coli*

5 First of all, an appropriate transformation-competent *E. coli* strain, which chromosomally codes a T7-RNA polymerase, is transformed with the expression plasmid manufactured under Step a), and subsequently incubated overnight at 30°C on culture medium solidified with agar. Suitable expression strains are, for example, BL21 strains (Invitrogen Prod. No.: C6010-03, which chromosomally code a T7-RNA  
10 polymerase under the control of an IPTG-inducible promoter (lacZ). Bacteria colonies resulting from the transformation can be investigated using methods known to the person skilled in the art to see whether they contain the required expression plasmid containing a cDNA coding the starch-phosphorylating protein. At the same time, expression clones are obtained.

15

c) Expression of a starch-phosphorylating protein in *Escherichia coli*

First, a preparatory culture is prepared. To do this, an expression clone obtained in accordance with Step b) is seeded in 30 ml Terrific Broth (TB medium) containing an antibiotic for selection on the presence of the expression plasmid, and incubated  
20 overnight at 30°C under agitation (250 rpm).

Next, a main culture is prepared for the expression of a starch-phosphorylating protein. To do this, in each case, 1 litre Erlenmeyer flasks, each containing 300 ml of TB medium, pre-heated to 30°C, and an antibiotic for selection on the presence of the expression plasmid are each seeded with 10 ml of an appropriate preparatory  
25 culture and incubated at 30°C under agitation (250 rpm) until an optical density (measured at a wavelength of 600 nm; OD<sub>600</sub>) of ca. 0.8 is achieved.

If, for the expression of a starch-phosphorylating protein, an expression plasmid is used, in which the expression of the starch-phosphorylating protein is initiated by means of an inducible system (e.g. the expression vector pDEST™17 in BL21 *E. coli*  
30 strains, inducible by means of IPTG), then on reaching an OD<sub>600</sub> of ca. 0.8, the inductor concerned (e.g. IPTG) is added to the main culture. After adding the inductor, the main culture is incubated at 30°C under agitation (250 rpm) until an OD<sub>600</sub> of ca. 1.8 is achieved. The main culture is then cooled for 30 minutes on ice

before the cells of the main culture are separated from the culture medium by centrifugation (10 minutes at 4.000xg and 4°C).

#### 4. Purification of a Starch-phosphorylating Protein

##### 5 a) Breaking down of cells expressing a starch-phosphorylating protein

The cells obtained in Step c), General Methods, Item 3 are re-suspended in lysis buffer. In doing so, ca. 4 ml lysis buffer are added to about 1 g of cells. The re-suspended cells are then incubated for 30 minutes on ice before they are broken down with the help of an ultrasonic probe (Baudelin Sonoplus UW 2070, Baudelin electronic; Berlin, settings: Cycle 6, 70%, 1 minute) under continuous cooling by means of the ice. Care must be taken here to ensure that the cell suspension is not heated too much during the ultrasonic treatment. The suspension obtained after the ultrasonic treatment is centrifuged (12 minutes at 20.000xg, 4°C) and the supernatant obtained after centrifugation is filtered using a filter with a pore size of 45 µm.

15

##### b) Purification of the starch-phosphorylating protein

If the starch-phosphorylating protein expressed in *E. coli* cells is a fusion protein with a His tag, then cleaning can take place with the help of nickel ions, to which the His tag bonds with greater affinity. To do this, 25 ml of the filtrate obtained in Step d) is mixed with 1 ml Ni-agarose slurry (Qiagen, Prod. No.: 30210) and incubated for 1 hour on ice. The mixture of Ni-agarose slurry and filtrate is subsequently spread over a polystyrene column (Pierce, Prod. No.: 29920). The product, which runs through the column, is discarded. The column is next washed by adding 8 ml of lysis buffer, the product, which runs through the column, again being discarded. Elution of the starch-phosphorylating protein then takes place by fractionated addition to the column of 1 ml E1 buffer twice, followed by 1 ml E2 buffer once and subsequently 1 ml E3 buffer five times. The product, which runs through the column, which is produced by adding the individual fraction of the appropriate elution buffer (E1, E2, E3 buffer) to the column, is collected in separate fractions. Aliquots of these fractions are subsequently analysed by means of denaturing SDS acrylamide gel electrophoresis followed by Coomassie Blue colouring. The fractions, which contain the starch-phosphorylating protein in sufficient quantity and satisfactory purity, are

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cleaned and concentrated with the help of pressurised filtration at 4°C. Pressurised filtration can be carried out, for example, with the help of an Amicon cell (Amicon Ultrafiltration Cell, Model 8010, Prod. No.: 5121) using a Diaflo PM30 membrane (Millipore, Prod. No.: 13212) at 4°C. Other methods known to the person skilled in the art can also be used for concentration however.

c) Composition of buffers used

Lysis buffer: 50 mM HEPES

300 mM NaCl

10 mM Imidazole

pH 8.0 (adjust with NaOH)

1 mg/ml Lysozyme (add immediately before using the buffer)

¼ tablet per 10 ml protease inhibitors completely EDTA free, (Roche product No.: 1873580, add immediately before using the buffer)

Elution buffer E1: 50 mM HEPES

300 mM NaCl

50 mM Imidazole

pH 8.0 (adjust with NaOH)

Elution buffer E2: 50 mM HEPES

300 mM NaCl

75 mM Imidazole

pH 8.0 (adjust with NaOH)

Elution buffer E3: 50 mM HEPES

300 mM NaCl

250 mM Imidazole

pH 8.0 (adjust with NaOH)

## 5. Recombinant Expression of an R1 Protein

The recombinant expression of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the  
5 recombinant expression of a starch-phosphorylating protein described above under General Methods, Item 3.

## 6. Purification of an R1 Protein

Purification of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99,  
10 7166-7171; Mikkelsen et al., Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the cleaning of a starch-phosphorylating protein described above under General Methods, Item 4 if an R1 fusion protein, which contains a His tag, is produced by expression of R1 in *E. coli* cells.

15

## 7. In-vitro Manufacture of Phosphorylated Starch on the Basis of Non-Phosphorylated Starch

### a) *In vitro* phosphorylation of non-phosphorylated starch

Starch, which does not contain starch phosphate (e.g. isolated from leaves of  
20 *Arabidopsis thaliana* *sex1-3* mutants with the help of the methods described above under General Methods, Item 2), is mixed with R1 buffer and with purified R1 protein (ca. 0.25 µg R1 protein per mg starch) in order to produce a starch content of 25 mg per ml. This reaction preparation is incubated overnight (approx. 15 hours) at room temperature under agitation. R1 bonded to the starch present in the reaction  
25 preparation is removed on completion of the reaction by washing four times with ca. 800 µl 0.5 % SDS in each case. Subsequently, the SDS still present in the *in vitro* phosphorylated starch is removed by washing five times with 1 ml wash buffer in each case. All washing steps take place at room temperature for 10 to 15 minutes under agitation. Each washing step is followed by a centrifugation (2 min, 10.000xg),  
30 in order to separate the starch granules from the respective SDS buffer or wash buffer.

b) Composition of buffers used

R1 buffer:	50 mM	HEPES/KOH,	pH	7.5
	1 mM	EDTA		
5	6 mM	MgCl <sub>2</sub>		
	0.5 mM	ATP		

Wash buffer: 50 mM HEPES/KOH, pH 7.2

10 **8. Bonding of Proteins to Phosphorylated Starch and Non-Phosphorylated Starch**

a) Isolation of P-starch protein complexes or non-phosphorylated starch protein complexes

Ca. 50 mg P-starch or ca. 50 mg non-phosphorylated starch respectively are re-  
15 suspended in separate preparations in ca. 800 µl protein extract in each case. The  
protein concentration of the protein extracts should be ca. 4 mg to 5 mg per ml in  
each case. Incubation is carried out on the P-starch or non-phosphorylated starch  
with protein extracts for 15 minutes under agitation at 4°C. On completion of the  
incubation, the reaction preparations are centrifuged out using a Percoll cushion (4  
20 ml) (15 minutes, 3500 rpm, 4°C). Proteins, which are not bonded to phosphorylated  
starch or to P-starch, are located in the supernatant after centrifugation, and they can  
be removed using a Pasteur pipette. The supernatant is discarded. The sedimented  
pellet containing P-starch and non-phosphorylated starch, including the proteins  
bonded to the respective starches (P-starch protein complexes or non-  
25 phosphorylated starch protein complexes respectively), obtained after centrifugation  
is washed twice with 1 ml of wash buffer in each case (see above, General Methods  
under Item 7b) by incubating for 3 minutes at 4°C in each case under agitation. Every  
washing step is followed by a centrifugation (5 minutes, 8000 rpm, 4°C in a table  
centrifuge, Hettich EBA 12R) in order to separate the P-starch or non-phosphorylated  
30 starch respectively from the wash buffer.

b) Dissolving the proteins bonded in the P-starch protein complexes or non-phosphorylated starch protein complexes respectively

The P-starch protein complexes or non-phosphorylated starch protein complexes obtained according to Step a) are re-suspended in approx. 150 µl SDS test buffer in each case, and incubated for 15 minutes under agitation at room temperature. The P-starch or non-phosphorylated starch respectively is subsequently removed from the dissolved proteins by centrifugation (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge). The supernatant obtained after centrifugation is centrifuged again in order to remove all residue of P-starch or non-phosphorylated starch (1 minute, 13,000 rpm, room temperature, Eppendorf table centrifuge), and then it is removed. As a result, dissolved proteins, which bond to the P-starch or non-phosphorylated starch respectively, are obtained.

c) Composition of buffers used

15	SDS test buffer:	187.5 mM	Tris/HCl pH 6,8
		6 %	SDS
		30 %	Glycerine
		~ 0.015 %	Bromphenol blue
		60 mM	Dithioerythritol (DTE, add fresh!)

20

Percoll: Percoll is dialysed overnight against a solution consisting of [missing word?] and 25 mM HEPES / KOH, pH 7.0

## 25 9. Separation of Proteins that Bond to P-Starch and/or Non-Phosphorylated Starch

The dissolved proteins obtained in Step c) under General Methods, Item 8 relating to the bonding of proteins to P-starch or non-phosphorylated starch respectively are incubated for 5 minutes at 95°C in each case and subsequently separated with the help of denaturing polyacrylamide gel electrophoresis. In doing so, an equal volume is applied to the acrylamide gel in each case for the dissolved proteins obtained by bonding to P-starch and for those obtained by bonding to non-phosphorylated starch. The gel obtained on completion of electrophoresis is stained at least overnight with

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colloidal Comassie (Roth, Karlsruhe, Roti-Blue Rod. No.: A152.1), and subsequently de-stained in 30 % methanol, 5 % acetic acid or in 25% methanol.

## 10. Identification and Isolation of Proteins Bonding to P-Starch and/or Non-Phosphorylated Starch

a) Identification of proteins with increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch

Proteins, which, after separation by means of acrylamide gel electrophoresis and subsequent visualisation by colouration (see above, General Methods, Item 9),

exhibit an increased signal after bonding to P-starch in comparison with a corresponding signal after bonding to non-phosphorylated starch, have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch. By this means, it is possible to identify proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch. Proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch, are excised from the acrylamide gel.

Identification of the amino acid sequence of proteins, which have increased bonding activity with respect to P-starch in comparison with non-phosphorylated starch

Proteins identified in accordance with Step a) are digested with trypsin and the peptides obtained are analysed by means of MALDI-TOF to determine the masses of the peptides obtained. Trypsin is a sequence-specific protease, i.e. trypsin only splits proteins at a specified position when the proteins concerned contain certain amino acid sequences. Trypsin always splits peptide bonds when the amino acids arginine and lysine follow one another starting from the N-terminus. In this way, it is possible to theoretically determine all peptides that would be produced following the trypsin digestion of an amino acid sequence. From the knowledge of the amino acids coding the theoretically determined peptides, the masses of the peptides, which are obtained after theoretical trypsin digestion, can also be determined. Databases (e.g.

NCBI nr <http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>; Swissprot <http://cbrg.inf.ethz.ch/Server/MassSearch.html>), which contain information concerning the masses of peptides after theoretical trypsin digestion, can therefore

be compared with the real masses of peptides of unknown proteins obtained with MALDI-TOF-MS. Amino acid sequences, which have the same peptide masses after theoretical and/or real trypsin digestion, are to be looked upon as being identical. The databases concerned contain both peptide masses of proteins, the function of which has already been shown, and also peptide masses of proteins, which up to now only exist hypothetically by derivation from amino acid sequences starting from nucleic acid sequences obtained in sequencing projects. The actual existence and the function of such hypothetical proteins has therefore seldom been shown and, if there is a function at all, then this is usually based only on predictions and not on an actual demonstration of the function.

Bands containing proteins identified in accordance with Step a) are excised from the acrylamide gel; the excised acrylamide piece is reduced and destained by incubating for approximately half an hour at 37°C in ca. 1 ml 60% 50mM  $\text{NH}_4\text{HCO}_3$ , 40% acetonitrile. The decolourising solution is subsequently removed and the remaining gel dried under vacuum (e.g. Speedvac). After drying, trypsin solution is added to digest the proteins contained in the gel piece concerned. Digestion takes place overnight at 37°C. After digestion, a little acetonitrile is added (until the acrylamide gel is stained white) and the preparation is dried under vacuum (e.g. Speedvac). When drying is complete, just enough 5% formic acid is added to cover the dried constituents and they are incubated for a few minutes at 37°C. The acetonitrile treatment followed by drying is repeated once more. The dried constituents are subsequently absorbed in 0.1% TFA (trifluoroacetic acid, 5  $\mu\text{l}$  to 10  $\mu\text{l}$ ) and dripped onto a carrier in ca. 0.5  $\mu\text{l}$  portions. Equal amounts of matrix ( $\epsilon$ -cyano -4-hydroxycinnamic acid) are also applied to the carrier. After crystallising out the matrix, the masses of peptides are determined by means of MALDI-TOF-MS-MS (e.g. Bruker Reflex<sup>TM</sup> II, Bruker Daltonic, Bremen). With the masses obtained, databases are searched for amino acid sequences, which give the same masses after theoretical trypsin digestion. In this way, amino acid sequences can be identified, which code proteins, which preferably bond to phosphorylated alpha-1,4-glucans and/or which need P-alpha-1,4-glucans as a substrate.

## **11. Method for Demonstrating the Starch-phosphorylating Activity of A Protein**

### **a) Incubation of proteins with P-starch and/or non-phosphorylated starch**

In order to demonstrate whether a protein has starch-phosphorylating activity, proteins to be investigated can be incubated with starch and radioactively labeled ATP. To do this, ca. 5 mg of P-starch or ca. 5 mg of non-phosphorylated starch are incubated with the protein to be investigated (0.01 µg to 5.0 µg per mg of starch used) in 500 µl phosphorylation buffer for 10 minutes to 30 minutes at room temperature under agitation. The reaction is subsequently stopped by the addition of SDS up to a concentration of 2% (weight/volume). The starch granules in the respective reaction mixture are centrifuged out (1 minute, 13.000xg), and washed once with 900 µl of a 2 % SDS solution and four times each with 900 µl of a 2 mM ATP solution. Every washing step is carried out for 15 minutes at room temperature under agitation. After each washing step, the starch granules are separated from the respective wash buffer by centrifugation (1 min, 13.000xg).

In addition, when carrying out an experiment to demonstrate starch-phosphorylating activity of a protein, further reaction preparations, which do not contain protein or contain inactivated protein, but which are otherwise treated in the same way as the reaction preparations described, should be processed as so-called controls.

### **b) Determination of the amount of phosphate residues incorporated in the P-starch and/or non-phosphorylated starch due to enzymatic activity**

The starch granules obtained in accordance with Step a) can be investigated for the presence of radioactively labeled phosphate residues. To do this, the respective starch is re-suspended in 100 µl of water and mixed with 3 ml of scintillation cocktail in each case (e.g. Ready Safe™, BECKMANN Coulter) and subsequently analysed with the help of a scintillation counter (e.g. LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

### **c) Identification of proteins, which preferably use P-starch as a substrate**

If a protein is incubated in separate preparations, once with P-starch and once with non-phosphorylated starch, in accordance with the method described under a), then,

by comparing the values for the presence of starch phosphate obtained according to Step b), it can be determined whether the protein concerned has incorporated more phosphate in P-starch in comparison with non-phosphorylated starch. In this way, proteins can also be identified, which can introduce phosphate into P-starch but not into non-phosphorylated starch. That means proteins can be identified, which require already phosphorylated starch as a substrate for an additional phosphorylation reaction.

d) Composition of buffers used

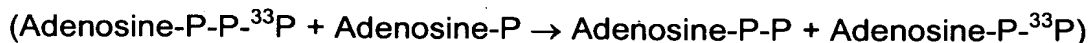
10 Phosphorylation buffer: 50 mM HEPES/KOH, pH 7.5  
1 mM EDTA  
6 mM MgCl<sub>2</sub>  
0.01 to 0.5 mM ATP  
0.2 to 2 µCi per ml randomised <sup>33</sup>P-ATP (alternatively,  
15 ATP, which contains a phosphate residue, which is specifically labeled in the beta position, can also be used)

In conjunction with the present invention, the term "randomised ATP" is to be understood to mean ATP, which contains labeled phosphate residues both in the gamma position and in the beta position (Ritte et al. 2002, PNAS 99, 7166-7171). Randomised ATP is also described in the scientific literature as beta/gamma ATP. A method for manufacturing randomised ATP is described in the following.

i) Manufacture of randomised ATP

The method described here for manufacturing randomised ATP with the help of enzyme-catalysed reactions is based on the following reaction mechanisms:

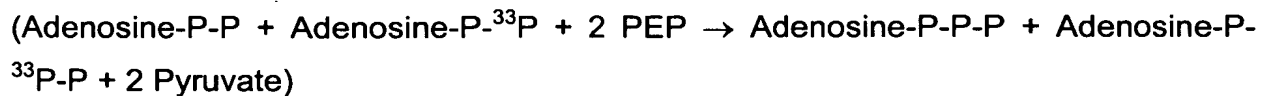
1st Reaction step:



30

2nd Reaction step:





The reaction equilibriums lie on the product side but, in spite of this, this reaction  
5 produces a mixture consisting mainly of  $\beta^{33}\text{P-ATP}$  and some  $\gamma^{33}\text{P-ATP}$ .

ii) Performing the first reaction step

ATP (100  $\mu\text{Ci}$ , 3000 Ci per mmol), which contains a phosphate residue labeled with  
 $^{33}\text{P}$  in the gamma position (Hartmann Analytic, 10  $\mu\text{Ci}/\mu\text{l}$ ), is incubated with 2  $\mu\text{l}$   
10 myokinase (AMP-phosphotransferase, from rabbit muscle; SIGMA, Prod. No.: M3003  
3.8 mg/ml, 1,626 units/mg) in 90  $\mu\text{l}$  randomising buffer for 1 hour at  $37^\circ\text{C}$ . The  
reaction is subsequently stopped by incubating for 12 minutes at  $95^\circ\text{C}$  before the  
reaction preparation is cleaned up by means of centrifugal filtration using a Microcon  
YM 10 filter (Amicon, Millipore Prod. No. 42407) at  $14.000\times g$  for at least 10 minutes.

15

iii) Performing the second reaction step

2  $\mu\text{l}$  pyruvate kinase (see below for how to manufacture an appropriate solution) and  
3  $\mu\text{l}$  50 mM PEP (phosphoenolpyruvate) are added to the filtrate obtained in Step ii).  
This reaction mixture is incubated for 45 minutes at  $30^\circ\text{C}$  before the reaction is  
20 stopped by incubating at  $95^\circ\text{C}$  for 12 minutes. The reaction mixture is subsequently  
centrifuged (2 minutes, 12,000 rpm in an Eppendorf table centrifuge). The  
supernatant containing randomised ATP obtained after centrifugation is removed,  
aliquoted and can be stored at  $-20^\circ\text{C}$ .

25 Producing the pyruvate kinase solution

15  $\mu\text{l}$  pyruvate kinase (from rabbit muscle, Roche, Prod. No. 12815), 10 mg/ml, 200  
units/mg at  $25^\circ\text{C}$ ) are centrifuged out, the supernatant is discarded and the pellet is  
absorbed in 27  $\mu\text{l}$  pyruvate kinase buffer.

iv) Buffers used

30 Pyruvate kinase buffer:    50 mM        HEPES/KOH pH 7.5  
   1 mM        EDTA

Randomising buffer:        100 mM        HEPES/KOH pH 7.5

	1 mM	EDTA
	10 %	Glycerol
	5 mM	MgCl <sub>2</sub>
	5 mM	KCl
5	0,1 mM	ATP
	0,3 mM	AMP

## 12. Demonstrating the Autophosphorylation of a Protein

In order to demonstrate whether a protein has auto-phosphorylating activity, proteins to be investigated can be incubated with radioactively labeled ATP. To do this, proteins to be investigated (50 µg to 100 µg) are incubated in 220 µl phosphorylation buffer (see above, Item 12 d), General Methods) for 30 minutes to 90 minutes at room temperature under agitation. The reaction is subsequently stopped by the addition of EDTA up to a final concentration of 0.11 M. Ca. 2 µg to 4 µg of protein are separated with the help of denaturing polyacrylamide gel electrophoresis (7.5% acrylamide gel). The gel obtained after polyacrylamide gel electrophoresis is subjected to autoradiography. Proteins, which exhibit a signal in the autoradiography, carry a radioactive phosphate residue.

## 13. Identification of the C-Atom Positions of the Glucose Molecules of an alpha-1,4-glucan, in which Residual Phosphates are Introduced through a Starch-phosphorylating Protein

Which C-atom positions of the glucose molecules of an alpha-1,4-glucan are phosphorylated by a protein can be demonstrated by hydrolysis of the phosphorylated glucan obtained by means of an appropriate protein *in vitro*, subsequent separation of the glucose monomers obtained after hydrolysis, followed by measurement of the phosphate incorporated by an appropriate protein in certain fractions of the glucose molecules.

a) Total hydrolysis of the alpha-1,4-glucans

Water suspensions containing alpha-1,4-glucan are centrifuged, the sedimented pellet subsequently re-suspended in 0.7 M HCl (Baker, for analysis) and incubated for 2 hours at 95°C under agitation. On completion of incubation, the samples are briefly cooled and centrifuged (e.g. 2 minutes 10.000xg). The supernatant obtained is transferred to a new reaction vessel and neutralised by the addition of 2 M NaOH (Baker, for analysis). If a pellet remains, it is re-suspended in 100 µl of water and the quantity of labeled phosphate present therein is determined as a control.

The neutralised supernatant is subsequently centrifuged over a 10-kDa filter. By measuring an aliquot of the filtrate obtained, the quantity of labeled phosphate in the filtrate is determined with the help of a scintillation counter, for example.

b) Fractionation of the hydrolysis products and determination of the phosphorylated C-atom positions

The neutralised filtrates of the hydrolysis products obtained by means of Step a) can be separated (when using radioactively labeled ATP about 3000 cpm) with the help of high-pressure anion exchange chromatography (HPAE), for example. The neutralised filtrate can be diluted with H<sub>2</sub>O to obtain the volume required for HPAE. In addition, glucose-6-phosphate (ca. 0.15 mM) and glucose-3-phosphate (ca. 0.3 mM) are added to the appropriate filtrates in each case as an internal control. Separation by means of HPAE can be carried out, for example, with the help of a Dionex DX 600 Bio Lc system using a CarboPac PA 100 column (with appropriate pre-column) and a pulsed amperometric detector (ED 50). In doing so, before injecting the sample, the column is first rinsed for 10 minutes with 99% eluent C and 1% eluent D. A sample volume of 60 µl is then injected. The elution of the sample takes place under the following conditions:

Flow rate: 1 ml per minute

Gradient: linearly increasing from 0 minutes to 30 minutes

	Eluent C	Eluent D
0 minutes	99%	1%
30 minutes	0%	100%
35 minutes	0%	100%
Run terminated		

The hydrolysis products eluted from the column are collected in individual fractions of 1 ml each. As, in each case, non-labeled glucose-3-phosphate (Ritte et al. 2002, PNAS 99, 7166-7171) and non-labeled glucose-6-phosphate (Sigma, Prod. No.: G7879) have been added to the injected samples of hydrolysis products as internal  
5 standards, the fractions, which contain either glucose-3-phosphate or glucose-6-phosphate, can be determined by means of pulsed amperometric detection. By measuring the amount of labeled phosphates in the individual fractions and subsequently comparing with the fractions, which contain glucose-3-phosphate or glucose-6-phosphate, this can be used to determine those fractions, in which labeled  
10 glucose-6-phosphate or labeled glucose-3-phosphate is contained. The amount of labeled phosphate in the fraction concerned is determined. From the ratios of the amounts of glucose-3-phosphate to glucose-6-phosphate measured for labeled phosphate in the individual hydrolysis products, it can now be determined which C-atom position is preferably phosphorylated by an alpha-1,4-glucan phosphorylating  
15 enzyme.

c) Buffers used:

Eluent C: 100 mM NaOH

Eluent D: 100 mM NaOH

20 500 mM sodium acetate

#### 14. Transformation of Rice Plants

Rice plants were transformed according to the method described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

25

#### 15. Transformation of Potato Plants

Potato plants were transferred with the help of agrobacterium, as described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).



## **16. Transformation of Wheat Plants**

Wheat plants were transformed according to the method described by Becker et al. (1994, Plant Journal 5, 299-307).

## **5 17. Transformation of Maize Plants**

Immature embryos of maize plants of line A188 were transformed according to the method described by Ishida et al. (1996, Nature Biotechnology 14, 745-750).

## **18. Determination of Starch Phosphate Content**

### **10 Determination of the C-6 phosphate content**

In the starch, the C2, C3, and C6 positions of the glucose units can be phosphorylated. For determination of the C6-P content of the starch, 50 mg of starch are hydrolysed in 500 µl 0.7 M HCl 4 h at 95°C. Subsequently, the preparations are centrifuged for 10 minutes at 15500 g, and the supernatant is removed. 7µl of  
15 supernatant is mixed with 193 µl imidazole buffer (100 mM imidazole, pH 7.4; 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0,4 mM NAD). The measurement was taken in the photometer at 340 nm. After establishing a base absorption, the enzyme reaction is started by adding two units of glucose-6-phosphate dehydrogenase (of *Leuconostoc mesenteroides*, Boehringer Mannheim). The change in absorption is directly  
20 proportional to the concentration of the G-6-P content in the starch.

### **b) Determination of the total phosphate content**

The determination of the total phosphate content occurs according to the Ames method (Methods in Enzymology VIII, (1966), 115-118).

25 Approximately 50 mg starch is mixed with 30 µl of ethanolic magnesium nitrate solution, and incinerated for three hours at 500°C in the muffle oven. The residue is mixed with 300 µl 0.5 M hydrochloric acid, and incubated for 30 minutes at 60°C. Subsequently, an aliquot is filled to 300 µl with 0.5 M hydrochloric acid, poured into a mixture of 100 µl 10% ascorbic acid and 600 µl 0.42% ammonium molybdate in 2 M  
30 sulphuric acid, and incubated for 20 minutes at 45°C.

c) Determination of the content of C-6 phosphate and C-3 phosphate

For the determination of the phosphate content, which is bonded in the C-6 position and the C-3 position of the glucose molecules of an alpha-1,4-glucan, the respective  
5 glucans can be separated using total hydrolysis according to the HPAE methods listed under General Methods 13. The quantities of glucose-6-phosphate and glucose-3-phosphate can be determined through integration of the individual peak areas obtained after HPEA separation. By comparing the peak surfaces obtained for  
10 glucose-6-phosphate in unknown samples with peak surfaces that were obtained after HPEA separation, having known quantities of glucose-6-phosphate and glucose-3-phosphate, the quantity of glucose-6-phosphate and glucose-3-phosphate can be determined in the samples to be examined.

15

## Examples

1. **Isolation of a protein from *Arabidopsis thaliana*, which exhibits increased bonding activity to P-starch in comparison to non-phosphorylated starch**

a) Manufacture of protein extracts from *Arabidopsis thaliana*

20 Protein extracts were produced from approximately 7 g of leaves (fresh weight) of *Arabidopsis thaliana* (Ökotyp Columbia, Col-O) according to General Methods, Item 1.

25 b) Isolation of starch granules from leaves of *sex1-3* mutants of *Arabidopsis thaliana*

Starch granules were isolated from about 20 g (fresh weight) of leaves of a *sex1-3* mutant of *Arabidopsis thaliana* according to the method described under General Methods, Item 2.

30 c) *In vitro* phosphorylation of starch, isolated from a *sex1-3* mutant of *Arabidopsis thaliana* with purified R1 protein

Approximately 30 mg of non-phosphorylated starch, isolated from a *sex1-3* mutant of *Arabidopsis thaliana*, was phosphorylated by way of an R1 protein recombinantly expressed and purified in *E. coli* according to the method described under General Methods, Item 7. For the expression of the R1 protein in *E. coli* and for subsequent  
5 purification, the method described by Ritte et al. (2002, PNAS 99, 7166-7171) was used.

d) Isolation of proteins, which bond to P-starch and/or non-phosphorylated starch  
Protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were  
10 incubated and washed in a Preparation A with 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) using the method described under General Methods, Item 8a.

In a second Preparation B, protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed with 50 mg of the non-  
15 phosphorylated starch manufactured in accordance with Step b) using the method described under General Methods, Item 8a.

Subsequently, the proteins bonded to the P-starch of Preparation A and to the non-phosphorylated starch of Preparation B were dissolved in accordance with the method described under General Methods, Item 8b.

20 In a third Preparation C, 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) were incubated and washed using the method described under General Methods, Item 8a. Preparation C contained no protein extracts however.

25 e) Separation of the proteins obtained in accordance with Step d) by means of acrylamide gel electrophoresis

The proteins of Preparations A, B and C obtained in Step d) were separated by means of a 9% acrylamide gel under denaturing conditions (SDS) using the method described under General Methods, Item 9, and subsequently stained with Coomassie  
30 Blue. The stained gel is shown in Fig. 1. It can be clearly seen that a protein, which has a molecular weight of ca. 130 kDa in denaturing acrylamide gel with regard to a protein standard marker (Trace M), preferably bonds to phosphorylated starch (Trace P) in comparison with non-phosphorylated starch (K).

f) Identification of the protein, which preferably bonds to P-starch in comparison with non-phosphorylated starch

The band of the protein with a molecular weight of ca. 130 kDa identified in Step e) was excised from the gel. The protein was subsequently released from the acrylamide as described under General Methods, Item 10b, digested with trypsin and the peptide masses obtained were determined by means of MALD-TOF-MS. The so-called "fingerprint" obtained by MALDI-TOF-MS was compared with fingerprints of theoretically digested amino acid molecules in databases (Mascot: [http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html); ProFound: [http://129.85.19.192/profound\\_bin/WebProFound.exe](http://129.85.19.192/profound_bin/WebProFound.exe); PepSea: <http://195.41.108.38/PepSeaIntro.html>). As such a fingerprint is very specific to a protein, it was possible to identify an amino acid molecule. With the help of the sequence of this amino acid molecule, it was possible to isolate a nucleic acid sequence from *Arabidopsis thaliana* coding an OK1 protein. The protein identified with this method was designated as A.t.-OK1. Analysis of the amino acid sequence of the OK1 protein from *Arabidopsis thaliana* showed that this deviated from the sequence that was present in the database (NP 198009, NCBI). The amino acid sequence shown in SEQ ID No 2 codes the A.t.-OK1 protein. SEQ ID No 2 contains deviations when compared with the sequence in the database (Acc.: NP 198009.1, NCBI). The amino acids 519 to 523 (WRLCE) and 762 to 766 (VRARQ) contained in SEQ ID No 2 are not in the sequence, which is present in the database (ACC.: NP 198009.1). Compared with Version 2 of the database sequence (Acc.: NP 198009.2), the amino acid sequence shown in SEQ ID NO 2 also contains the additional amino acids 519 to 523 (WRLCE).

## 2. Cloning a cDNA, which codes the identified OK1 protein

The A.t.-OK1 cDNA was isolated with the help of reverse PCR using mRNA isolated from leaves of *Arabidopsis thaliana*. To do this, a cDNA Strand was synthesised by means of reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT PCR, Invitrogen Prod. No.: 11904-018), which was then amplified using DNA polymerase (Expand High Fidelity PCR Systems, Roche Prod. No.: 1732641). The amplified product obtained from this PCR reaction was cloned in the vector pGEM®-T

(Invitrogen Prod. No.: A3600). The plasmid obtained is designated A.t.-OK1-pGEM, the cDNA sequence coding the A.t.-OK1 protein was determined and is shown under SEQ ID NO. 1.

5 The sequence shown under SEQ ID NO 1 is not the same as the sequence, which is contained in the database. This has already been discussed for the amino acid sequence coding an A.t.-OK1 protein.

Conditions used for the amplification of the cDNA coding the A.t.-OK1 protein

First strand synthesis:

10 The conditions and buffer specified by the manufacturer were used. In addition, the reaction preparation for the first strand synthesis contained the following substances:

3 µg Total RNA

5 µM 3'-Primer (OK1rev1: 5'-GACTCAACCACATAACACACAAAGATC)

0.83 µM dNTP Mix

15 The reaction preparation was incubated for 5 minutes at 75°C and subsequently cooled to room temperature.

The 1<sup>st</sup> strand buffer, RNase inhibitor, and DTT were then added and incubated for 2 minutes at 42°C before 1 µL Superscript RT DNA polymerase was added and the reaction preparation was incubated for 50 minutes at 42°C.

Conditions for the amplification of the first strand by means of PCR:

20 1 µL of the reaction preparation of the first strand synthesis

0.25 µM 3'Primer (OK1rev2: 5'- TGGTAACGAGGCAAATGCAGA)

0.25 µM 5'Primer (OK1fwd2: 5'- ATCTCTTATCACACCACCTCCAATG)

Reaction conditions:

Step 1 95°C 2 min

25 Step 2 94°C 20 sec

Step 3 62°C 30 sec (Temp. per cycle—0.67°C) (30 s), 68°C (

Step 4 68°C 4 minutes

Step 5 94°C 20 sec

Step 6 56°C 30 sec

30 Step 7 68°C 4 minutes

Step 8 68°C 10 minutes

The reaction was first carried out in accordance with Steps 1 to 4. 10 repeats (cycles) were carried out between Step 4 and Step 2, the temperature of Step 3 being

reduced by 0.67°C after each cycle. This was subsequently followed by the reaction in accordance with the conditions specified in Steps 5 to 8. 25 repeats (cycles) were carried out between Step 7 and Step 5, the time of Step 7 being increased by 5 sec on each cycle. On completion of the reaction, the reaction was cooled to 4°C.

5    **3.    Creation of a vector for recombinant expression of the cDNA of the OK1 protein**

Following amplification by means of PCR by using the plasmid A.t.-OK1-pGEM as a template using Gateway Technology (Invitrogen), the sequence coding the OK1 protein from *Arabidopsis thaliana* was next cloned in the vector pDONOR™ 201  
10 (Invitrogen Prod. No.: 11798-014). pDONOR™ 201. Subsequently, the coding region of the OK1 protein from the vector obtained was cloned by sequence-specific recombination in the expression vector pDEST17™ (Invitrogen Prod. No.: 11803-014). The expression vector obtained was designated as A.t.-OK1-pDEST™17. The cloning resulted in a translational fusion of the cDNA coding the A.t.-OK1 protein with  
15 the nucleotides present in the expression vector pDEST™17. The nucleotides originating from the vector pDEST™17, which are translationally fused with the cDNA coding the A.t.-OK1 protein, code 21 amino acids. These 21 amino acids include, amongst others, the start codon (ATG) and a so-called His tag (6 histidine residues directly after one another). After translation of these translationally fused sequences,  
20 this results in an A.t.-OK1 protein, which has the additional 21 amino acids coded by nucleotides originating from the vector at its N-terminus. The recombinant A.t.-OK1 protein resulting from this vector therefore contains 21 additional amino acids originating from the vector pDEST™17 at its N-terminus.

25    **4.    Heterologous expression of the OK1 protein in E. coli**

The expression vector A.t.-OK1-pDEST™17 obtained in accordance with Example 3 was transformed in the *E. coli* strain BL21 Star™ (DE3) (Invitrogen, Prod. No. C6010-03). A description of this expression system has already been given above (see General Methods, Item 3). Bacteria clones, containing the vector A.t.-OK1-  
30 pDEST™17, resulting from the transformation were next used to manufacture a

preparatory culture, which was subsequently used for inoculating a main culture (see General Methods, Item 3c). The preliminary culture and the main culture were each incubated at 30°C under agitation (250 rpm). When the main culture had reached an OD<sub>600</sub> of ca. 0.8, the expression of the recombinant A.t.-OK1 protein was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) until a final concentration of 1 mM was achieved. After the addition of IPTG, the main culture was incubated at 30°C under agitation (250 rpm) until an OD<sub>600</sub> of ca. 1.8 was achieved. The main culture was then cooled for 30 minutes on ice before the cells of the main culture were separated from the culture medium by centrifugation (10 minutes at 4.000xg and 4°C).

#### **5. Purification of the recombinantly expressed OK1 protein**

The purification and concentration of the A.t.-OK1 protein from cells obtained in accordance with Example 4 was carried out using the method described under General Methods, Item 4.

#### **6. Demonstration of starch-phosphorylating activity of the OK1 protein**

The starch-phosphorylating activity of the A.t.-OK1 protein was demonstrated in accordance with the method described under General Methods, Item 11. In doing so, 5 µg of cleaned A.t.-OK1 Protein manufactured in accordance with Example 5 was in each case incubated in a Preparation A with 5 mg of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with Example 1b) and in a Preparation B with 5 mg of starch obtained by enzymatic phosphorylation in accordance with Example 1c), in each case in 500 µl of phosphorylation buffer containing 0.05 mM radioactively (<sup>33</sup>P) labeled, randomised ATP (in total 1,130,00 cpm, ca. 0.55 µCi) for 30 minutes at room temperature under agitation. A Preparation C was used as a control, which was the same as Preparation B, except that it contained no OK1 protein, but was otherwise treated in the same way as Preparations A and B. Two tests, which were independent from one another, were carried out for all preparations (A, B, C).

Using a scintillation counter, the starches from Preparations A, B, and C were investigated for the presence of radioactively labeled phosphate (see General Methods, Item 11b). The results are shown in Table 1 and in Fig. 3.

5

	Measured radioactivity [cpm]	
	Trial 1	Trial 2
Preparation A (non-phosphorylated starch + OK1)	42	47
Preparation B (phosphorylated starch + OK1)	7921	8226
Preparation C (phosphorylated starch without protein)	56	53

Table 1: Demonstration of starch-phosphorylating activity of the OK1 protein

- 10 From the results obtained, it can be seen that the OK1 protein does not transfer phosphate groups from ATP to starch when non-phosphorylated starch is provided as a substrate, as the proportion of phosphate groups transferred to non-phosphorylated starch by means of an OK1 protein, measured in cpm, does not exceed the proportion of radioactively labeled phosphate groups in Preparation C
- 15 (control). If, on the other hand, P-starch is provided as a substrate, the proportion of radioactive phosphate groups, measured in cpm, which are transferred from ATP to P-starch, is significantly higher. From this, it can be seen that the OK1 protein requires P-starch as a substrate and that non-phosphorylated starch is not accepted as a substrate by the OK1 protein.
- 20 If the test described above is carried out with ATP specifically labeled in the gamma position with  $^{33}\text{P}$ , then it is not possible to establish an incorporation of radioactively labeled phosphate in the starch. From this, it can be seen that the beta phosphate residue of ATP is transferred from an OK1 protein to starch. The results of such a test are shown in Fig. 6.

25



## 7. Demonstration of autophosphorylation

Autophosphorylation of the A.t.-OK1 protein was demonstrated by means of the methods described above (see General Methods, Item 12). Here, 50 µg of purified A.t.-OK1 protein were incubated with radioactively labeled, randomised ATP in 220 µl of phosphorylation buffer (see above, General Methods, Item 12d) at room temperature for 60 minutes under agitation. Subsequently, 100 µl in each case were removed from the incubation preparations and transferred to four fresh reaction vessels. In reaction vessel 1, the reaction was stopped by the addition of 40 µl 0.11M EDTA. Reaction vessel 2 was incubated at 95°C for 5 minutes. HCl was added to reaction vessel 3 up to a final concentration of 0.5 M, and NaOH was added to reaction vessel 4 up to a final concentration of 0.5 M. Reaction vessels 3 and 4 were each incubated for 25 minutes at 30°C. Subsequently, 50 µl in each case were removed from reaction vessels 1, 2, 3 and 4, mixed with SDS test buffer and separated by means of SDS acrylamide gel electrophoresis (7.5% acrylamide gel). For this purpose, samples from the reaction vessels were applied to each of two identical acrylamide gels. One of the gels obtained on completion of electrophoresis was subjected to autoradiography, while the second gel was stained with Coomassie Blue.

In the gel stained with Coomassie Blue (see Fig. 2A), it can be clearly seen that treatment with 0.5 M NaOH leads to a degradation of OK1 protein. The OK1 protein must therefore be described as unstable compared with NaOH. Incubations at 30°C, 95°C and with 0.5 M HCl show that the OK1 protein is relatively stable under the stated incubation conditions. This can be concluded from the fact that, under these incubation conditions, in each case approximately the same amounts of OK1 protein can be demonstrated in the gel concerned after colouring with Coomassie Blue.

In the autoradiography (see Fig. 2B), it can be seen by comparison with the phosphorylated OK1 protein incubated at 30°C that an incubation of the phosphorylated OK1 protein at 95°C leads to a significant reduction in the phosphate, which has bonded to the OK1 protein. The bond between the phosphate residue and an amino acid of the OK1 protein must therefore be described as heat-unstable. Furthermore, a slight reduction of the phosphate bonded to the OK1 protein can also be seen for the incubation with 0.5 M HCl and 0.5 M NaOH in comparison with

phosphorylated OK1 protein incubated at 30°C. If the fact is taken into account that the quantity of OK1 protein in the autoradiography after treatment with 0.5 M NaOH is significantly less than in the samples treated with heat and acid on account of the instability of the OK1 protein compared with NaOH, then it can be concluded that the bond between the phosphate residue and an amino acid of the OK1 protein will be relatively stable with respect to bases. As the sample treated with acid contains approximately the same amounts of protein as the sample incubated at 30°C and at 95°C, and yet has a significantly lower signal in the autoradiography than the sample treated at 30°C, it must be assumed that acid incubation conditions also split the bond between a phosphate residue and an amino acid of the OK1 protein to a certain extent. An instability in the bond between a phosphate residue and an amino acid of the OK1 protein could therefore also be established in the tests carried out. At the same time, the instability with respect to acids is significantly less labeled than the instability with respect to heat.

Bonds between the amino acids histidine and phosphate are heat-unstable, acid-unstable but base-stable (Rosenberg, 1996, Protein Analysis and Purification, Birkhäuser, Boston, 242-244). The results described above are therefore an indication that a phosphohistidine is produced by the autophosphorylation of an OK1 protein.

If recombinantly expressed OK1 protein, as described above, is incubated with ATP specifically labeled with  $^{33}\text{P}$  in the gamma position, then no autophosphorylation can be detected. Fig. 5A shows the amount of protein in the respective reaction preparation that can still be demonstrated by means of Western blot analysis after the appropriate incubation steps. Fig. 5B shows an autoradiography of protein from the individual reaction preparations. It can be seen that, when ATP specifically labeled in the gamma position is used, no autophosphorylation of the OK1 protein takes place, whereas, when randomised ATP is used, autophosphorylation can be demonstrated. This means that when an OK1 protein is autophosphorylated, the phosphate residue of the beta position of the ATP is covalently bonded to an amino acid of the OK1 protein.

## 8. Demonstration of the C-atom positions of the glucose molecules of starch phosphorylated by an OK 1 protein

### a) Manufacture of phosphorylated starch

Phosphorylated starch was manufactured in accordance with General Methods, Item 7. For this purpose, 5 mg non-phosphorylated starch, isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* was reacted with 25 µg purified A.t.-OK1 protein in a Preparation A, and 5 mg *in vitro* phosphorylated starch, originally isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana*, was reacted with 5 µg purified R1 protein in a second Preparation B. In each case, the reaction occurred in 500 µl phosphorylation buffer, which contained <sup>33</sup>P labeled ATP in each case (ca. 2.5 x 10<sup>6</sup> cpm), by way of incubation at room temperature for 1 hour under agitation. In addition, a control preparation was used, which contained 5 mg of starch isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* and the said phosphorylation buffer, but no protein. The control preparation was treated in exactly the same way as Preparations A and B. The individual reactions were stopped by adding 125 µl 10% SDS in each case and washed with 900 µl in each case, once with 2% SDS, five times with 2 mM ATP and twice with H<sub>2</sub>O. A centrifugation was carried out after each washing step (2 minutes in an Eppendorf table centrifuge at 13,000 rpm in each case). The starch pellets obtained were re-suspended in 1 ml H<sub>2</sub>O in each case, 100 µl of each preparation was mixed after adding 3 ml of scintillation cocktail (Ready Safe<sup>TM</sup>, BECKMANN), and the preparations were subsequently measured with the aid of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER<sup>TM</sup>).

The measurement provided the following results:

Control:	63 cpm/100 µL	630 cpm/1000 µl
Preparation A (OK1):	1351 cpm/100 µl	13512 cpm/1000 µl
Preparation B (R1):	3853 cpm/100 µl	38526 cpm/1000 µl

### b) Total hydrolysis of the P-starch

The suspensions of Preparations A, B and C obtained in accordance with Step a) were centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), the pellets obtained re-suspended in 90 µl 0.7 M HCl (Baker, for analysis) and

subsequently incubated for 2 hours at 95°C. Preparations A, B and C were then centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), and the supernatant transferred to a new reaction vessel. Sedimented residues of the preparations were re-suspended in 100 ml H<sub>2</sub>O in each case and after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) were measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™). Significant amounts of radioactivity could not be demonstrated in any of the residues, which mean that all the hydrolysis products labeled with radioactive phosphate were located in the supernatant.

- 10 This was followed by neutralisation of the individual supernatants containing the hydrolysis products by the addition in each case of 30 µl 2 M NaOH (the amount of NaOH required for neutralisation was tested out in advance on blind samples): The neutralised hydrolysis products were placed on a 10 kDa Microcon filter, which had previously been rinsed twice with 200 µl H<sub>2</sub>O in each case, and centrifuged for ca. 25 minutes at 12,000 rpm in an Eppendorf table centrifuge. 10 µl were taken from the filtrate obtained (ca. 120 µl in each case) and, after the addition of 3 ml of scintillation cocktail (Ready Safe™, BECKMANN), were measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™). The determination of the activity present in the individual preparations gave the following results:

Preparation A (OK1):	934 cpm/10 µl	11,208 cpm/120 µl	93 cpm/µl
Preparation B (R1):	2518 cpm/10 µl	30,216 cpm/120 µl	252 cpm/µl

c) Separation of the hydrolysis products

- 25 The hydrolysis products obtained in accordance with Step b) were separated by means of HPAE using a Dionex system under the conditions stated above (see General Methods, Item 13c). The samples for separating the filtered supernatants of Preparations A and B obtained in accordance with Step b) were composed as follows:
- 30 Preparation A (OK1): 43 µl of the supernatant of Preparation A obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 32 µl H<sub>2</sub>O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Σ Volume = 80 µl).

Preparation B (R1): 16 µl of the supernatant of Preparation B obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 59 µl H<sub>2</sub>O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Σ Volume = 80 µl).

In each case, 60 µl, containing ca. 3,000 cpm, of the appropriate samples were  
5 injected for separation by means of HPAE. The HPAE was carried out in accordance with the conditions specified under Item 23c. After passing through the HPAE column, the elution buffer was collected in fractions, each of 1 ml. Collection of the fractions was begun 10 minutes after injecting the sample. Based on the signal received from the PAD detector used, the elution of glucose-6-phosphate was  
10 assigned to fraction 15 and the elution of glucose-3-phosphate to fraction 17. In each case, 500 µl of the individual fractions were mixed with 3 ml of scintillation cocktail (Ready Safe™, BECKMANN) and subsequently measured with the help of a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™). The following measurements were obtained for the individual  
15 fractions:

	Total cpm per Fraction	
	Preparation A (OK1)	Preparation B (R1)
Fr 13	8.7	3.3
Fr 14	13.1	32.2
Fr 15 (G6P)	207.3	1952.8
Fr 16	399.8	112.3
Fr 17 (G3P)	1749.2	801.6
Fr 18	196.7	17.3
Fr 19	6.7	18.9
Total	2581.5	2938.3
Deposit	3000.0	3000.0
Recovery	86.0%	97.9%

**Table 4:** Measured amounts of radioactivity [cpm] in individual fractions of hydrolysis products obtained by hydrolysis of starch phosphorylated by means of an OK1 protein or R1 protein.

The results are also shown graphically in Fig. 5.

After phosphorylation of starch catalysed by R1 protein, ca. 66% of the radioactively labeled phosphate, with respect to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction, which contained glucose-6-phosphate as standard, and ca. 27% with the fraction, which contained glucose-3-phosphate as standard. After phosphorylation of starch catalysed by OK1 protein, ca. 67% of the radioactively labeled phosphate, with respect to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction, which contained glucose-3-phosphate as standard, and ca. 8% with the fraction, which contained glucose-6-phosphate as standard. From this, it can be concluded that glucose molecules of the starch of R1 proteins are preferably phosphorylated in the C-6 position, whereas from OK1 proteins glucose molecules of the starch are preferably phosphorylated in the C-3 position.

## 9. Identification of an OK1 protein in rice

Using the methods described under General Methods, Items 1 to 13, it was also possible to identify a protein from *Oryza sativa* (variety M202), which transfers a phosphate residue from ATP to P-starch. The protein was designated as O.s.-OK1. Non-phosphorylated starch is not used by the O.s.-OK1 protein as a substrate, i.e. the O.s.-OK1 protein also does not need P-starch as a substrate. The nucleic acid sequence defining the identified O.s.-OK1 protein is shown under SEQ ID NO 3 and the amino acid sequence coding the O.s.-OK1 protein is shown under SEQ ID NO. 4. The amino acid sequence coding the O.s.-OK1 protein shown under SEQ ID NO 4 has an identity of 57% with the amino acid sequence coding the A.t.-OK1 protein shown under SEQ ID NO 2. The nucleic acid sequence coding the O.s.-OK1 protein shown under SEQ ID NO 3 has an identity of 61% with the nucleic acid sequence coding the A.t.-OK1 protein shown under SEQ ID NO 1.

Manufacture of the plasmid pMI50 containing the nucleic acid sequence coding an OK1 protein from *Oryza sativa*

The vector pMI50 contains a DNA fragment, which codes the complete OK1 protein from rice of the variety M202.

The amplification of the DNA from rice was carried out in five sub-steps.

The part of the open reading frame from position 11 to position 288 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os\_ok1-R9 (GGAACCGATAATGCCTACATGCTC) and Os\_ok1-F6 (AAACTCGAGGAGGATCAATGACGTCGCTGCGGCCCTC) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML123.

The part of the open reading frame from position 250 to position 949 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os\_ok1-F4 (CCAGGTTAAGTTTGGTGAGCA) and Os\_ok1-R6 (CAAAGCACGATATCTGACCTGT) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML120.

The part of the open reading frame from position 839 to position 1761 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os\_ok1-F7 (TTGTTCGCGGGATATTGTCAGA) and Os\_ok1-R7 (GACAAGGGCATCAAGAGTAGTATC) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML121.

The part of the open reading frame from position 1571 to position 3241 of the sequence specified under SEQ DIE NO 3 was amplified with the help of reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os\_ok1-F8 (ATGATGCGCCTGATAATGCT) and Os\_ok1-R4 (GGCAAACAGTATGAAGCACGA) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML119.

The part of the open reading frame from position 2777 to position 3621 was amplified with the help of polymerase chain reaction using the synthetic oligonucleotides Os\_ok1-F3 (CATTGGATCAATGGAGGATG) and Os\_ok1-R2 (CTATGGCTGTGGCCTGCTTTGCA) as a primer on genomic DNA of rice. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML122.

The cloning together of the sub-parts of the open reading frame of OK1 was carried out as follows.

A 700 base pair along *Apal* fragment of pML120, containing part of the open reading frame of OK1, was cloned in the *Apal* site of pML121. The plasmid obtained was designated as pMI47.

A 960 base pair long fragment containing the areas of vectors from pML120 and pML123 coding for OK1 was amplified by means of polymerase chain reaction. In doing so, the primers Os\_ok1-F4 (see above) and Os\_ok1-R9 (see above), each in a concentration of 50 nm, and the primers Os\_ok1-F6 and Os\_ok1-R6, each in a concentration of 500 nm, were used. The amplified DNA fragment was cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pMI44.

An 845 base pair long fragment of pML122 was re-amplified for introducing a *Xho*I site after the stop codon with the primers Os\_ok1-F3 (see above) and Os\_ok1-R2Xho (AAAACTCGAGCTATGGCTGTGGCCTGCTTTGCA) and cloned in the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as t pMI45.

A 1671 base pair long fragment containing part of the open reading frame of OK1 was obtained from pML119 by digesting with the restriction enzymes *Spe*I and *Pst*I. The fragment was cloned in pBluescript II SK+ (Genbank Acc.: X52328). The plasmid obtained was designated as pMI46.

A 1706 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Spe*I and *Xho*I from pMI46 and cloned in the vector pMI45, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI47.



A 146 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Afl*II/*Not*I from pMI43 and cloned in the vector pMI44, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI49.

- 5 A 1657 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Not*I and *Nar*I from the vector pMI49 and cloned in the vector pMI47, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI50 and contains the whole coding region of the OK1 protein identified in rice.

10

#### **10. Production of an antibody, which specifically recognises an OK1 protein**

As an antigen, ca. 100 µg of purified A.t.-OK1 protein was separated by means of SDS gel electrophoresis, the protein bands containing the A.t.-OK1 protein were excised and sent to the company EUROGENTEC S.A. (Belgium), which carried out  
15 the manufacture of the antibody under contract. Next, the preimmune serums of rabbits were investigated to see whether they would already detect a protein from an A. t. total extract before immunisation with recombinant OK1. The preimmune serums of two rabbits detected no proteins in the range 100-150 kDa and were thus chosen for immunisation. 4 injections of 100 µg of protein (Tag 0, 14, 28, 56) were given to  
20 each rabbit. 4 blood samples were taken from each rabbit: (Tag 38, Tag 66, Tag 87 and the final bleeding). Serum, obtained after the first bleeding, already showed a specific reaction with OK1 antigen in Western blot. However, in all further tests, the last bleeding of a rabbit was used.

#### **25 11. Production of transgenic rice plants, which exhibit increased activity of an OK1 protein**

##### **a) Manufacture of the plasmid pGlo-A.t.-OK1**

The plasmid pIR94 was obtained by amplifying the promoter of the globulin gene from rice by means of a polymerase chain reaction (30 x 20 sec 94 °C, 20 sec 62 °C,  
30 1 min 68 °C, 4 mM Mg<sub>2</sub>SO<sub>4</sub>) with the primers glb1-F2 (AAAACAATTGGCGCCTGGAGGGAGGAGA) and glb1-R1

(AAAACAATTGATGATCAATCAGACAATCACTAGAA) on the genomic DNA of rice of the variety M202 with High Fidelity Taq Polymerase (Invitrogen, catalogue number 11304-011) and cloned in pCR2.1 (Invitrogen catalogue number K2020-20).

The plasmid pIR115 was obtained by cloning a synthetic piece of DNA consisting of

5 the two oligonucleotides X1

(TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACACTAGTAAGCTTAATTAAGAT  
ATCATTTAC) and X2

(AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCT  
CTGCAGCCTGCA) in the vector pGSV71 excised with *Sdal* and *MunI*.

10 The plasmid pIR115 obtained was excised with *Sdal*, the protruding 3'-ends smoothed with T4 DNA polymerase, and a *HindIII* / *SphI* fragment from pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230) with a size of 197 base pairs, smoothed by means of T4 DNA polymerase and containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted.

15 The plasmid obtained was designated as pIR96.

The plasmid pIR103 was obtained by cloning a 986 base pair long DNA fragment from pIR94 containing the promoter of the globulin gene from rice, which was cloned in the plasmid pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which is derived from the intermediate  
20 vector pGSV1. pGSV1 constitutes a derivative of pGSC1700, the construction of which has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene and deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

25 pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) as well as the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene *aadA*, from the transposon Tn1331 from *Klebsiella pneumoniae*, which gives resistance against the antibiotics spectinomycin and streptomycin (Tolmasky,  
30 Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40).

The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the promoter sequence of

the cauliflower mosaic virus for initiating the transcription (Odell et al., Nature 313, (1985), 180), the *bar* gene from *Streptomyces hygrosopicus* (Thompson et al., Embo J. 6, (1987), 2519-2523) and the 3'-untranslated area of the nopaline synthase gene of the T-DNA of pTiT37 for terminating the transcription and polyadenylation.

- 5 The *bar* gene provides tolerance against the herbicide glufosinate ammonium.

A DNA fragment, which contains the sequence of the entire open reading frame of the OK1 protein from *Arabidopsis*, was excised from the vector A.t.-OK1-pGEM, and cloned into the vector pIR103. For this purpose, the plasmid A.t.-OK1-pGEM was excised with the restriction enzyme *Bsp120I*, the ends were smoothed with T4-DNA  
10 polymerase, and re-excised with *Sall*. The DNA fragment coding the OK1 protein from *Arabidopsis thaliana* was cloned into the vector pIR103, which was excised with *Ecl136II* and *XhoI*. The plasmid obtained was designated as pGlo-A.t.-OK1.

b) Transformation of rice plants

- 15 Rice plants (variety M202) were transformed with *Agrobacterium* (containing the plasmid pGlo-A.t.-OK1), using the method described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

c) Analysis of the transgenic rice plants and the starch synthesised from these

- 20 By way of quantitative RT PCR analysis, it was possible to identify plants, which exhibit an expression of mRNA coding A.t.-OK1 protein.

Plants, which exhibit a detectable amount of mRNA coding A.t.-OK1 protein in comparison to corresponding wild type plants, were grown in the greenhouse. Grains of these plants were harvested. Starch, isolated from these mature grains, showed  
25 an increased content of phosphate covalently bonded to the respective starch in comparison to starch, which was isolated from grains of corresponding wild type plants.

## 12. Production of transgenic potato plants, which exhibit increased activity of an OK1 protein

### a) Manufacture of the plasmid pBinB33-Hyg

Starting with the plasmid pBinB33, the EcoRI-HindIII fragment containing the B33 promoter, a part of the polylinker, and the ocs-terminator were excised and spliced into the correspondingly excised vector pBIB-Hyg (Becker, 1990, Nucl. Acids Res. 18, 203). The plasmid pBinB33 was obtained by splicing the promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989) as a *DraI* fragment (nucleotide - 1512 - +14) into the vector pUC19 excised with *SstI*, the ends of which had been smoothed with the help of the T4 DNA polymerase. This resulted in the plasmid pUC19-B33. The B33 promoter was excised from this plasmid with *EcoRI* and *SmaI* and spliced into the correspondingly excised vector pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230). This resulted in the plant expression vector pBinB33.

15

### b) Manufacture of the vector A.t.-OK1-pBinB33-Hyg

The coding sequence of the A.t.-OK1 protein was excised with the restriction endonucleases Bsp120I and Sall from the plasmid A.t.-OK1-pGEM and spliced into the vector pBinB33-Hyg excised with *SmaI* and *Sall*. The plasmid obtained was designated as A.t.-OK1-pBinB33-Hyg.

20

### c) Transformation of potato plants

*Agrobacterium tumefaciens* (strain GV2260) was transformed with the plasmid A.t.-OK1-pBinB33-Hyg. Subsequently, potato plants of the variety Désirée were transformed with the help of the *Agrobacterium tumefaciens* containing the plasma A.t.-OK1-pBinB33-Hyg in accordance with the method described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29), and the plants were regenerated. The plants obtained from this transformation event were designated 385JH.

25

### 30 d) Analysis of the transgenic potato plants and the starch synthesised from these

By means of Northern Blot analysis, it was possible to identify plants of the transformation event 385JH, which exhibit an expression of mRNA, coding the A.t.-OK1 protein.

A Western Blot analysis, which was performed with the antibody described under Example 10, confirmed, that plants of the transformation event 385JH, in which mRNA of the heterologously expressed OK1 protein was detected, also exhibit an increased quantity of OK1 protein in comparison to wild type plants that have not been transformed. Fig. 7 exemplary shows the detection of the A.t.-OK1 Protein in single plants from the transformation event 385JH by means of Western Blot analysis. For induction of the B33 Promotor in leaf tissue single lines of the the transformation event 385JH were cultivated on solidified Musharige Skoog medium containing 100 mM sucrose in tissue culture for two days. After harvest protein extracts were produced from leaf tissue of these plants according to the method described under General Methods, Item 1a). After separation of the proteins by means of denaturing polyacrylamide gel electrophoreses 40 µg protein extract of each line was analysed by means of Western Blot analysis using the antibody described under Examples, Item 10. As control samples, protein extracts from Arabidopsis plants and from potato wildtype plants (cv Désirée) were also analysed. Plants, which exhibit an increased quantity of OK1 protein and a detectable quantity of A.t.-OK1 protein coding mRNA, were grown in the greenhouse. Starch, which was isolated from tubers of these plants, showed an increased content of phosphate bonded covalently to the corresponding starch.

### 13. Production of transgenic maize plants, which exhibit increased activity of an OK1 protein

#### a) Manufacture of the plasmid pUbi-A.t.-OK1

First the plasmid pIR96 was manufactured. The plasmid pIR96 was obtained by cloning a synthetic piece of DNA consisting of the two oligonucleotides X1 (TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACTAGTAAGCTTAATTAAGAT ATCATTAC) and X2 (AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCT CTGCAGCCTGCA) into the vector pGSV71 excised with *SdaI* and *MunI*. The

plasmid obtained was excised with *SdaI* and the protruding 3'-ends were smoothed with T4 DNA polymerase. The plasmid obtained was excised with *SdaI*, the protruding 3'-ends were smoothed with T4 DNA polymerase, and a 197 base pair large *HindIII* / *SphI* fragment from pBinAR, smoothed with T4 DNA polymerase  
5 (Höfgen und Willmitzer, 1990, Plant Science 66, 221-230), and containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted. The plasmid obtained was designated as pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which is derived from the intermediate vector pGSV1. pGSV1 constitutes a derivative of pGSC1700, the construction of  
10 which has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene and deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2,  
15 (1977), 95-113) as well as the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene *aadA*, from the transposon Tn1331 from *Klebsiella pneumoniae*, which gives resistance against the antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40).

20 The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the promoter sequence of the cauliflower mosaic virus for initiating the transcription (Odell et al., Nature 313, (1985), 180), the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al., Embo J. 6, (1987), 2519-2523) and the 3'-untranslated area of the nopaline synthase  
25 gene of the T-DNA of pTiT37 for terminating the transcription and polyadenylation. The *bar* gene provides tolerance against the herbicide glufosinate ammonium.

A 1986 base pair long fragment containing the promoter of the polyubiquitin gene from maize (Genes from Maize (Gens aus Mais) (EMBL Acc.: 94464, Christensen et al., 1992, Plant Mol. Biol. 18: 675-689) was cloned as a *PstI* fragment into  
30 pBluescript II SK+. The plasmid obtained was designated as pSK-ubq.

The plasmid A.t.-OK1-pGEM was excised with the restriction enzyme *Bsp120I*, the ends were smoothed with T4-DNA polymerase, and it was re-excised with *SacI*. The DNA fragment coding the OK1 protein from *Arabidopsis thaliana* was cloned into the

plasmid pSK-ubq, which was excised with *Sma*I and *Sac*I. The plasmid obtained was designated as pSK-ubq-ok1.

A fragment was isolated from the plasmid pSK-ubq-ok1, which contains the ubiquitin promoter from maize and the entire open reading frame for the A.t.-OK1 protein from *Arabidopsis thaliana*. For this purpose, the plasmid was excised with the restriction enzyme *Asp*718I, the ends were filled with T4 DNA polymerase, and it was re-excised with *Sda*I. The 5799 base pair large fragment obtained was cloned into the plasmid pIR96 excised with *Eco*RV and *Pst*I. The plasmid obtained from this cloning was designated as pUbi-A.t.-OK1.

10

b) Transformation of maize plants

Maize plants were transformed with the plasmid pUbi-A.t.-OK1 using the method described under General Methods, Item 17.

- 15 c) Analysis of the transgenic maize plants and the starch synthesised from these  
Using Northern Blot analysis, plants could be identified, which exhibit an expression of mRNA, coding the A.t.-OK1 protein.

Maize plants, which exhibit a detectable amount of A.t.-OK1 protein coding mRNA in comparison to corresponding wild type plants, were grown in the greenhouse. Single grains of these plants were harvested. Starch, isolated from these grains, showed an increased content of phosphate covalently bonded to the respective starch in comparison to starch, which is isolated from grains of corresponding wild type plants.

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**14. Manufacture of transgenic wheat plants, which exhibit increased activity of an OK1 protein**

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a) Manufacture of a plasmid for the transformation of wheat plants

pMCS5 (Mobitec, [www.mobitec.de](http://www.mobitec.de)) was digested with *Bgl*II and *Bam*HI and re-inserted. The plasmid contained was designated as pML4.

The *nos* terminator from *Agrobacterium tumefaciens* (Depicker et al., 1982, Journal of Molecular and Applied Genetics 1: 561-573) was amplified with the primers P9 (ACTTCTgCAgCggCCgCgATCgTTCAAACATTTggCAATAAAgTTTC) and P10 (TCTAAgCTTggCgCCgCTAgCAgATCTgATCTAgTAACATAgATgACACC) (25

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cycles, 30 sec 94 °C, 30 sec 58 °C, 30 sec 72 °C), digested with *HindIII* and *PstI*, and cloned into the plasmid pML4 having been excised with the same enzymes. The plasmid contained was designated as pML4-nos. A 1986 base pair long fragment containing the promoter of the polyubiquitin gene from maize (Genbank Acc.: 94464, Christensen et al., 1992, Plant Mol. Biol. 18: 675-689) and the first intron of the same gene, shortened through digestion by *Clal* and re-insertion, were cloned into this vector. The plasmid contained was designated as pML8.

The entire open reading frame of OK1 from *Arabidopsis thaliana* was cloned into the plasmid pML8. In order to this, the corresponding fragment with *Bsp120/NotI* was excised from A.t.-OK1-pGEM, and spliced into the *NotI* site of pML8 in an „in sense „ orientation.

A fragment for the transformation of wheat plants can be excised from the obtained vector pML8-A.t.-OK1 with the restriction enzymes *AvrII* and *SwaI*, which contains the promoter of the polyubiquitin gene from maize, the entire open reading frame of OK1 from *Arabidopsis thaliana*, and the *nos* terminator from *Agrobacterium tumefaciens*.

#### b) Transformation of wheat plants

Wheat plants of the Florida variety were transformed with a fragment purified from an agarose gel, which was excised with the restriction enzymes *AvrII* and *SwaI* from the plasmid pML8-A.t.-OK1, and which contains the promoter of the polyubiquitin gene from maize, the entire open reading frame of OK1 from *Arabidopsis thaliana*, and the *nos* terminator from *Agrobacterium tumefaciens*, and with the plasmid pGSV71 using the biolistic method according to the method described by Becker et al. (1994, Plant Journal 5, 299-307).

c) Analysis of the transgenicwheat plants and the starch synthesised from these Starch was isolated from mature grains of the T0 plants obtained from the transformation, and the content of phosphate covalently bonded to the starch was determined. The phosphate content of the starch, which was isolated from individual grains, was clearly higher than in the case of the starch, which was isolated from grains of corresponding wild type plants.